Characterisation of a subset of *Escherichia coli* river isolates and their role in the environmental resistome

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A thesis submitted to the University of Warwick for the degree of

Doctor of Philosophy

University of Warwick, School of Life Sciences

June 2020

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Acknowledgments

I would like to express my deep gratitude to **Professor Elizabeth Wellington** for her always kind support and guidance while navigating these four years of research together. She gave me the freedom and trust to lead my work, allowing me to grow as a scientist. I am also grateful for all the opportunities she offered me throughout the Ph.D. to learn and develop my career so positively.

I thank **Professor Peter Hawkey** for his inspiring career that paved the way for many of us in the field of AMR. I am grateful for the help and expertise he provided me throughout my research.

My gratitude goes to the members of my advisory panel, **Professor Yin Chen** and **Dr Hendrik Schäfer**. Their encouraging words and insightful comments on my research never failed to help me when I needed it.

I thank **Dr Gavin Collins** for his kind help, expertise on anaerobic digester and for welcoming me at The National University of Ireland, Galway.

I would like to thank all past and present members of the Wellington's Lab for all their help, advice and encouragement during the course of this work. In particular, I thank **Dr Jennifer Holden** for the incredible warmth, kindness and patience she showed me when working together. I am grateful for everything she taught me in the lab but more importantly, I am thankful for her friendship and all of the joyful moments we have spent together. I also thank **Dr Chiara Borsetto**, an amazing scientist and an incredible friend, who have helped me immensely during all of the stages of my Ph.D. I am thankful for the support and help she gave me in the lab. We have faced all of the ups and downs of my research together and they were, after all, quickly forgotten with her. Thank you to **Dr Ian Lidbury** for the help, guidance and clarity he brought me for my project. I have learned so much from all of his advice and I am grateful that he was willing to push me when I needed it the most. I thank **Dr Robert James** for initiating me to the magic of long-read sequencing. I am thankful for his expertise and

the endless amount of time he spent helping and supporting me, especially during the writing of my thesis. I would also like to thank **Andrew Murphy** for always being present to help and advise me in the lab. A warm thank you to **Dr Rui Abreu Pereira** for his support and expertise of research. His inimitable sense of humour and sarcasm that I grew to love, always brought laughter and smile to my lunch breaks. I thank **Dr Sébastien Raguideau** for his assistance with all of the bioinformatics analyses. Sharing a cup of tea, a biscuit and one of his unparalleled jokes with him made the bioinformatics less intimidating.

A special thanks also to the dear friends I met throughout this journey. First, a warm thank you to the inspiring **Audam Chhun**, the best flatmate and thoughtful friend that I could ever dream of. Friends like him are very rare and I cannot thank him enough for the incredible support, comfort, light and joy he brought to this English adventure. I am very grateful that proteomics brought us together. Second, *merci* to the members of the French team – **Dr Arnaud Kengmo Tchoupa**, **Dr Agnès Kuroki** and **Dr Sandra Bedarida** - for all those cheerful and unique moments we have spent together; this adventure would have not been the same without them. A particular and loving thought for Sandra, for who I am thankful for always being there for me and for her incredible moral support. Third, thanks to my dear friend **Betty Sands** (LB), a caring and graceful soul who never fails to warm my heart while on our crazy adventures together. I also thank **Linda Westermann**, **Sireethorn Tungsirisurp**, **Eleonora Silvano**, **Holly Shropshire**, **Letizia Pondini**, **Richard Guillonneau**, **Vinko Zadjelovic**, **Gabriel Erni Cassola**, **Aurore Lison and Valentin Waschulin** with who I shared incredible moments in and outside University. Last but not least, thank you to my lifelong friends **Fabrice Donz** and **Emmanuelle Donz** for always supporting me but also for making sure I remain crazy; **Jimena Santiago, Laura Boutet**, **Flora Peillard**, **Chloé Aymard**, **Clément Danis** and **Sami Rouahi** for the love, encouragement and for making my time at university unforgettable.

Finally, thank you to every member of **my family** for making me feel supported and loved without fail since I left. The list is far too long to name each person individually, but I know you will know who you are.
To my beloved parents**, Claudéus** and **Lolita** and amazing brother, **Zinédine**, thank you for believing in me in times where my confidence was wavering; thank you for

your unconditional love and support that brought me comfort even through my hardest days; and thank you for making me feel home while 10,000 kilometres away.

I dedicate this thesis to **Suzie**, **Adrienne** and **Cina**, thank you for being so kind and loving to me.

Declaration

In accordance with the University of Warwick regulations for the degree of Doctor of Philosophy, I certify that this thesis has been written solely by me. The work contained in this thesis is my own unless otherwise stated. No aspect of this work has been submitted to any other institution for any other degree of award.

Abstract

Infections caused by antimicrobial resistant bacterial pathogens are fast becoming an important global health issue. Our research group have identified wastewater treatment plants (WWTPs) as hotspots for the dissemination of antimicrobial resistant genes/bacteria (ARG/ARB) into the environment. Specifically, strains of *E. coli* ST131 carrying multiple resistance genes including *bla*$_{\text{CTX-M-15}}$ (encoding extended spectrum beta-lactamase, ESBL).

Previous work produced isolates from a local river which included *E. coli* ST131 strain 48 downstream of a WWTP and harbouring *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA}}$ and *bla*$_{\text{TEM}}$ in its genome. Through conjugation with a donor strain the plasmid was isolated and sequenced and found to harbour the *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM}}$. The *de novo* assembly of the plasmid was achieved using long-reads sequences by using a new bioinformatic pipeline and showed the *bla*$_{\textit{CTX-M-15}}$ was the only resistance gene located on this plasmid.

The data revealed that the strain 48 expressed and secreted an ESBL that provided a protective effect against cefotaxime for susceptible cells. *In silico* analysis predicted a signal peptide for CTX-M-15 and TEM. Exoproteomics and whole-cell proteomics identified CTX-M-15 as the major secreted ESBL, confirming *in silico* analysis. Heterologous expression of the *bla*$_{\text{CTX-M-15}}$ gene in another *E. coli* strain confirmed that this gene was responsible for the observed protective effect against cefotaxime. It becomes clear that the plasmid pSRJ48c was highly beneficial to strain 48 and persisted in absence of selection indicated a balance between plasmid cost and benefit. An evolutionary hypothesis on the co-evolution of host and plasmid support these observations.

The survival of strain 48 under prolonged anaerobic digestion in the presence and absence of a third-generation cephalosporin was investigated and provided evidences of the survival of the *E. coli* 48 and the persistence of *bla*$_{\text{CTX-M-15}}$ through a nine-day anaerobic incubation. The sensitive commensal strain failed to survive both in presence and the absence of the antibiotic. The survival of 48 poses multiple risks to human health as this strain has the potential to be the host for other ARGs and can contribute to the spread of ARGs.

Overall, the work developed in this thesis suggests that more rigorous controls of AD parameters should be employed to reduce survival of ARB. Further work could lead to the optimisation of the process for AD both for human and animal waste.

Abbreviations

AB	Anaerobic bioreactor
AD	Anaerobic digestion
AMR	Antimicrobial resistant
APEC	Avian pathogenic *E. coli*
ARB	Antimicrobial resistant bacteria
ARG	Antimicrobial resistant gene
AS	Activated sludge
ASVs	Amplicon sequence variants
bp	Base pair
CM	Condition medium
CSTR	Continuously stirred tank reactor
Ct	Cycle threshold
CTX-M	Cefotaxime- Munich
d	day
DAEC	Diffusively adherent E. coli
DCS	Dairy cattle-slurry
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
eDNA	Cell-free DNA
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasice *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ESBL	Extended spectrum β-lactamase
ExPEC	Extraintestinal pathogenic *E. coli*
FOG	Fats, oils, grease
GIT	Gastrointestinal tract
Gsp	General secretion pathway
HGT	Horizontal gene transfer
HMW	High molecular weight

ICE	Integrative conjugative elements
IMP	Imipenamase
Inc	Incompatibility
IS	Insertion sequences
KO	Knock-out
LB	Luria Bertani
MBL	Metallo-β-lactamase
MDR	Multidrug resistant
MGE	Mobile genetic element
MOB	Mobility
MLST	Multilocus sequence typing
MPN	Most Probable Number
NDM-1	New Delhi metallo-β-lactamase 1
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling
NMEC	Neonatal meningitis *E. coli*
OMVs	Outer-membrane vesicles
o/n	Overnight
ONT	Oxford Nanopore Technologies
ORF	Open reading frame
oriT	Origin of transfer
OTU	Operational taxonomic units
Oxacillinase	OXA
PacBio	Pacific Biosciences
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Rep	Replication initiation
RNA	Ribonucleic acid
RND	Resistance nodulation division
Sec	General secretory pathway
SEPEC	Septicaemia associated *E. coli*
ST	Sequence type

T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SS	Type 6 secretion system
TA	Toxin-antitoxin
Ta	Annealing temperature
Tat	Twin-arginine pathway
TC	Transconjugant
Tn	Transposon
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
UK	United Kingdom
UV	Ultraviolet
VFs	Virulence factors
VIM	Verona integron-encoded MBL
WGS	Whole Genome Sequencing
WWTP	Wastewater treatment plant

Chapter 1:

Introduction

1.1 The species *Escherichia coli*

1.1.1 A brief overview of *E. coli*

Escherichia coli (*E. coli*) is a motile Gram-negative bacterium, facultative anaerobes part of the *Enterobacteriaceae* family, it has been the subject of a number of studies and become the best-characterised prokaryotic organisms (Kaper *et al.*, 2004). *E. coli* is generally considered to be a commensal species that lives in the gastrointestinal tract (GIT) of humans and animals. However, through the acquisition of virulence factors (VFs) some *E. coli* strains are considered as either intestinal or extraintestinal pathogens (Croxen and Finlay, 2010).

1.1.2 *E. coli* typing

The identification of a clone depends on the molecular typing technique used. The most commonly used for *E. coli* is multilocus sequence typing (MLST). Seven housekeeping genes are targeted and a concatemer of the sequence is used to interrogate a sequence type (ST) database (Maiden *et al.*, 1998; Clermont *et al.*, 2015). The MLST allele sequences and ST profile table are stored in curated databases. There are currently three MLST databases for *E. coli*, the one hosted by the Michigan State University, USA, was initially developed for enteropathogenic *E.* coli, whereas the one hosted by the Warwick Medical School, UK and Pasteur Institute, France were not focused on any particular group (Reid *et al.*, 2000; Wirth *et al.*, 2006; Jaureguy *et al.*, 2008).

Another approach for typing has been to use next-generation sequencing (NGS) and obtain high-resolution sequence data which can be used in combination with bioinformatic tools for *in silico* analysis. This method offers a high resolution and allows discrimination between highly related strains and the phylogenetic relationship between a group of strains (Uelze *et al.*, 2020). The initial approach of the MLST

using the seven housekeeping genes is now expanded to core genome MLST which compares genomes by using a large number of gene loci (Maiden *et al.*, 1998; Maiden *et al.*, 2013). There are now a number of well-designed and implemented tools such as Enterobase that allow visualization of groupings from whole-genome sequencing (WGS) to define ST and also genomic relationships and core genome diversity within the genus (Zhou *et al.*, 2019). WGS will become the gold standard for the identification clinically important strains in the future and allow surveillance for outbreaks (Pitout and DeVinney, 2017).

1.2 Clinical significance of *E. coli*

1.2.1 *E. coli* causing GIT disease

Six pathogenic categories of *E. coli* are able to cause intestinal disease, including the enteropathogenic *E. coli* (EPEC), the enterohaemorrhagic *E. coli* (EHEC), the enterotoxigenic *E. coli* (ETEC), the enteroaggregative *E. coli* (EAEC), the enteroinvasive *E. coli* (EIEC) and the diffusely adherent *E. coli* (DAEC). The diseases caused by these pathogens are summarised in Table 1.1.

Table 1.1 GIT diseases caused by *E. coli*.

Pathogen	Diseases caused
EPEC	Severe diarrhoea and predominantly in children mainly in developed countries (Ochoa and Contreras, 2011)
EHEC	Bloody and non-bloody diarrhoea when present in recreational and municipal drinking water, or following consumption of undercooked meats or unpasteurized milk (Kaper *et al.*, 2004)
ETEC	Watery diarrhoea in the developing world due to the production of a heat stable enterotoxins (Nataro and Kaper, 1998; Chaudhuri and Henderson, 2012)
EAEC	Persistent diarrhoea in both adults and children in developing and developed countries
EIEC	Causes watery diarrhoea and might cause an invasive inflammatory colitis (Nataro and Kaper, 1998)
DAEC	Diarrhoeal disease in particularly in children over 12 months of age (Nataro and Kaper, 1998)

1.2.2 Extra-intestinal pathogenetic *E. coli* (ExPEC)

Extra-intestinal pathogenic *E. coli* (ExPEC) represent the biggest burden of *E. coli* disease in the developed world, including the United Kingdom (UK). These strains can be found among the commensals of the gut and stably colonize the intestinal tract of the host without causing enteric disease (Wiles *et al.*, 2008). The ExPEC group include the uropathogenic *E. coli* (UPEC) which is the most prevalent cause of urinary tract infection (UTI), the neonatal meningitis *E. coli* (NMEC), the avian pathogenic *E. coli* (APEC) and the septicaemia associated *E. coli* (SEPEC) (Figure 1.1).

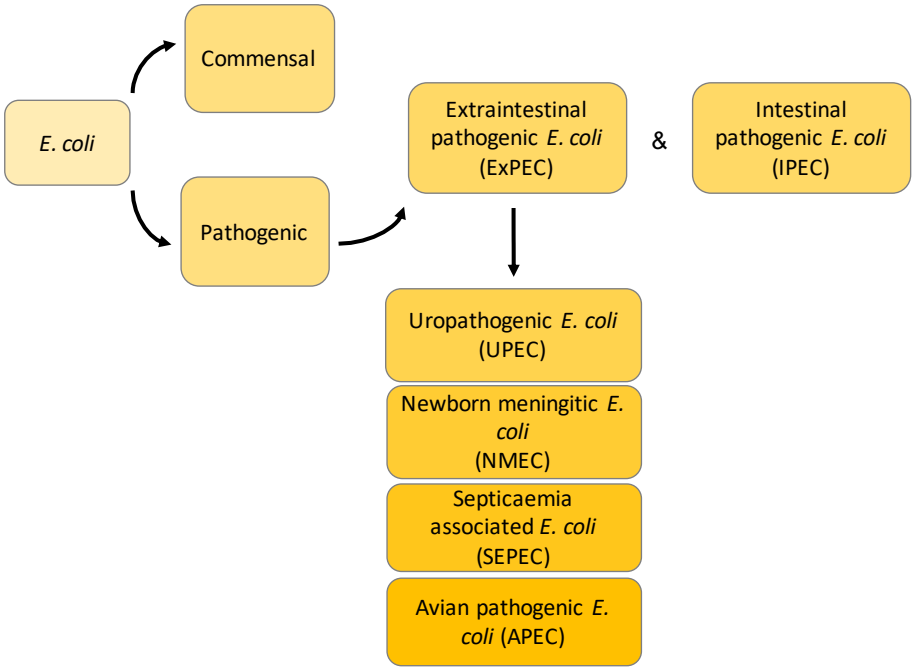


Figure 1.1 Extra-intestinal pathogenic *E. coli* (ExPEC) strain. Figure is adapted from (Sarowska *et al.*, 2019).

The predominant clonal group of ExPEC is the MLST type ST131 which has become globally disseminated (Price *et al.*, 2013).

ExPEC are associated with VFs such as adhesins, toxins, iron acquisition factors and invasins which are often encoded on pathogenicity islands and mobile genetic elements (MGEs) such as plasmids (Kohler and Dobrindt, 2011) (Table 1.2).

Table 1.2 Non-exhaustive list of virulence-associated factor present in ExPEC (Pitout, 2012; Sarowska *et al.*, 2019).

	Virulence factor
Adhesins	
F10 *papA*	P fimbriae subunit variant
papC	*papACEFG*, genes of P fimbriae operon
pap EF/G	*papACEFG*, genes of P fimbriae operon
sfa/foc	S or F1C fimbriae
focG	F1C fimbriae adhesin
iha	Adhesion siderophore
fimH	Type 1 fimbriae
tsh	Temperature sensitive hemagglutinin
hra	Heat-resistant agglutinin
afa/draBC	Dr-binding adhesins
TOXIN	
hlyD	α-Hemolysin
sat	Secreted autotransporter toxin
pic	Serine protease
vat	Vacuolating toxin
astA	Enteroaggregative *E. coli* toxin
cnf1	Cytotoxic necrotizing factor
SIDEROPHORES	
iroN	Salmochelin (siderophore) receptor
fyuA	Yersiniabactin (siderophore) receptor
ireA	Siderophore receptor
iutA	Aerobactin (siderophore) receptor
CAPSULE	
kpsM II	*kpsM* II group2capsule
K1	K1 group 2 capsule variants
K2	K2 group 2 capsule variants
K5	K5 group 2 capsule variants
kpsMT III	Group 3 capsule
MISCELLANEOUS	
usp	Uropathogenic-specific protein
traT	Serum resistance-associated
ompT	Outer membrane protease T
iss	Increased serum survival
H7 *fliC*	Flagellin variant
malX	Pathogenicity island marker

Half of *E. coli* community-onset bacteraemia occurred in patients with a history of healthcare interventions (e.g. urinary catheterisation, antibiotic therapy) and it is estimated that bacteraemia in England will increase by 22% by 2021 if no action is taken (Bhattacharya *et al.*, 2018). Presence of *E. coli* in the bloodstream triggers host inflammatory responses and can lead to organ failure.

1.3 Antimicrobial therapy

The advent of antibiotic chemotherapy changed the way in which bacterial pathogens impacted human population globally but also had a dramatic impact on the way in which farm animals were protected from infections. Unfortunately, most drug resistance has arisen from clinical exposure to antibiotic followed by dramatic dissemination *via* MGEs and the 'ESKAPE' describes the most frequent and challenging multidrug resistant (MDR) microorganisms; *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aerugionosa* and *Enterobacter* species (Rice, 2008; Khan and Khan, 2016) (Figure 1.2). Antimicrobial resistance (AMR) represents one of the most challenging threats to human health of the 21st century. As well as huge economic cost, it is estimated that by 2050, 10 million deaths a year could be due to AMR if no action is taken to slow down the rise of antimicrobial resistant bacteria (ARB) (O'Neill, 2016). Whilst much attention is now being turned towards understanding the drivers of AMR, research to date has primarily focused on analysing the effects of single variables on the environmental resistome, such as the impacts of farming, or wastewater treatment plant (WWTP) effluent. Questions remain as to how indigenous resistance genes transfer to pathogenic organisms, the selective pressures that drive these processes and the role of the environment in AMR dissemination (Wellington *et al.*, 2013).

A list of the most common antibiotics with their mode of actions and resistance mechanisms are shown in Table 1.3.

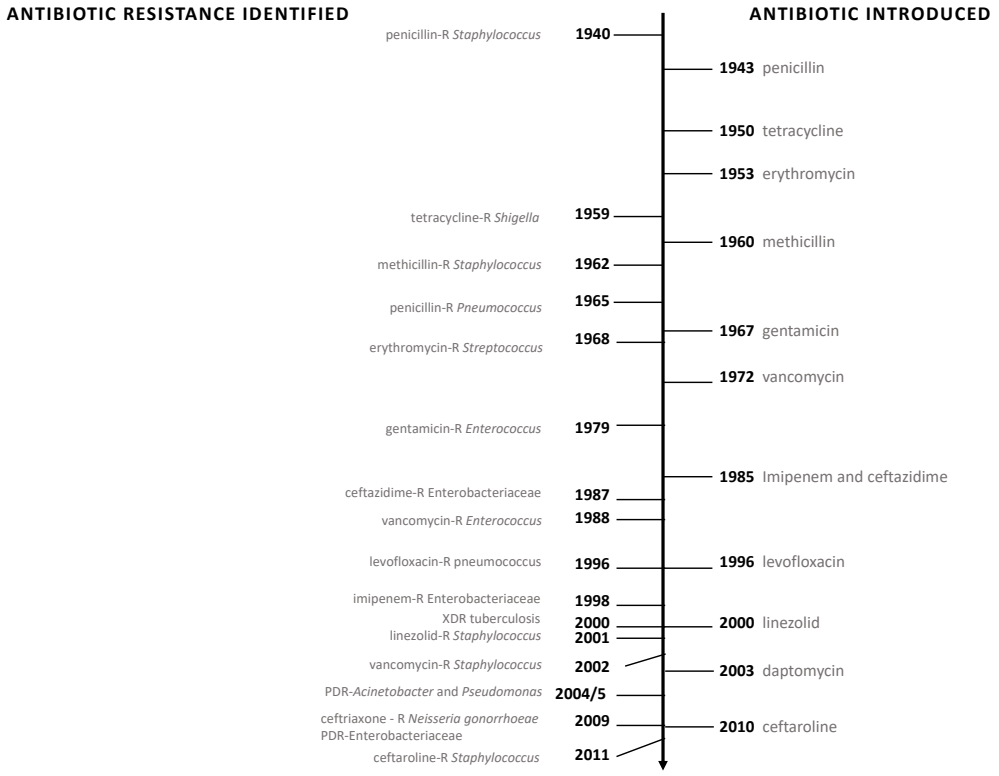


Figure 1.2 Timeline of antibiotic deployment and identification of corresponding resistance. Figure is adapted from (CDC, 2013).

Table 1.3 Modes of action and resistance mechanism of commonly used antibiotics (Davies and Davies, 2010; Lewis, 2013).

Antibiotic class	Example (s)	Mode of actions	Activity or target species	Mode(s) of resistance
β-lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Inhibition of cell wall biosynthesis	Broad-spectrum activities	Hydrolysis, efflux, altered target
Aminoglycosides	gentamicin, streptomycin, spectinomycin, kanamycin,	Binding of the 30S ribosomal subunit	Broad-spectrum activities	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Inhibition of cell wall biosynthesis	Gram-positive bacteria	Reprogramming peptidoglycan biosyntehsis
Tretracyclines	Minocycline, tetracycline	Binding of the 30S ribosomal subunit	Broad-spectrum activities	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Binding of the 50S ribosomal subunit	Broad-spectrum activities	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Oxazolidinones	Linezolid	Binding of the 50S ribosomal subunit	Gram-positive bacteria	Efflux, altered target
Phenicols	Chloramphenicols	Binding of the 50S ribosomal subunit	Broad-spectrum activities	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin, levofloxacin	Inhibition DNA synthesis	Broad-spectrum activity	Acetylation, efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism		Efflux, altered target
Lipopeptides	Daptomycin	Depolarization of cell membrane	Gram-positive bacteria	Altered target
Cationic peptides	Colistin	Cell membrane		Altered target, Efflux
Streptogramins	Synercid	Binding of the 50S ribosomal subunit	Gram-positive bacteria	C-O lyase (type B streptogramins), acetylation (type A streptogramins)

Historically, β-lactam antibiotics were used over the last 70 years for the treatment of *Enterobacteriaceae* following the advent of semi-synthesis from penems and cefems providing a multitude of sub-classes with a more broad-spectrum activity (Table 1.4). The continued development of semi-synthesis has generated multiple compounds with increased activity and resistance to β-lactamases that can be grouped from 1st generation cephalosporin to 4th generation as shown in Figure 1.3.

Table 1.4 β-lactam subclasses with examples of marketed antibiotics. Adapted from (Ribeiro da Cunha *et al.*, 2019).

Subclasses	Examples
Penicillins	Penicillin G, Penicillin V, Ampicillin, Amoxicillin, Bacampicillin, Cloxacillinm, Floxacillin, Mezlocillin, Nafcillin, Oxacillin, Methicillin a, Dicloxacillin, Carbenicillin, Idanyl, Piperacillin, Ticarcillin
Cephalosporins	Cefalothin, Cephradine, Cefadroxyl, Cefazolin, Cephalexin, Cefuroxine, Cefaclor, Cefotetam, Cefmetazole, Cefonicid, Cefixime, Ceftibuten, Cefizoxime, Ceftriaxone, Cefamandol, Cefoperazone, Cefotaxime, Proxetil, Cefprozil, Ceftazidime, Cefuroxime Axetil, Cefpodexime, Cefepime, Ceftobiprole
Carbapenems	Imipenem, Meropenem

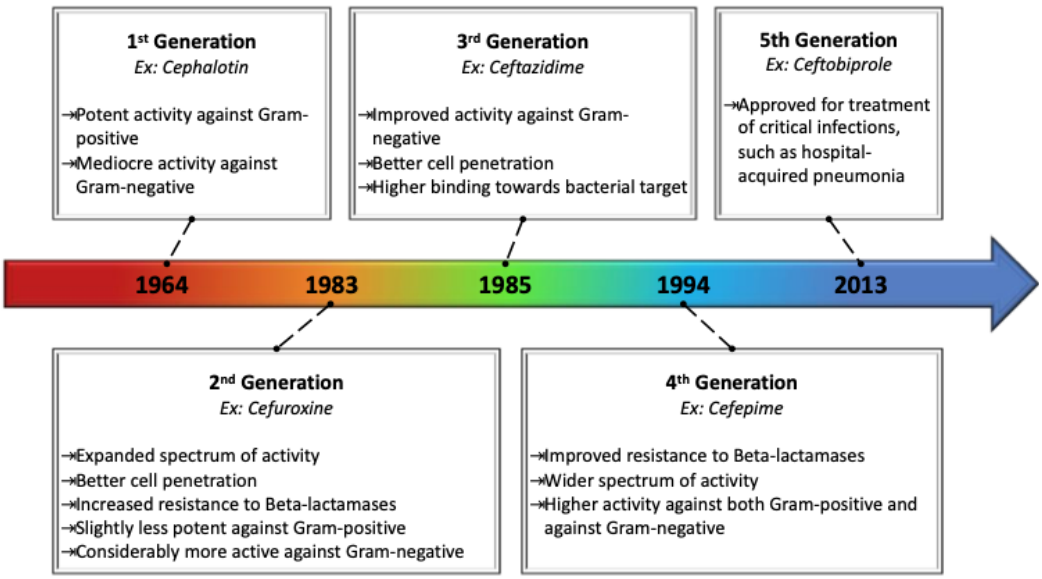


Figure 1.3 Evolution of cephalosporins over semi-synthetic generation. Each generation is the result of molecular addition groups at modifiable sites. Figure is extracted from (Ribeiro da Cunha *et al.*, 2019).

Gram-negative bacterial pathogen represents a more recalcitrant target than Gram-positive due to their complex cell-wall consisting of three layers; the outer membrane, the peptidoglycan cell wall and the inner membrane (Exner *et al.*, 2017; Breijyeh *et al.*, 2020). Thus, infection with MDR Gram-negative bacteria are common but with limited therapeutic option to treat them (Duval *et al.*, 2019; Ribeiro da Cunha *et al.*, 2019). This situation has forced clinicians to use old molecules, such as colistin, called a last-resort antibiotic but colistin-resistant Gram-negative bacteria have appeared all around the word (Jacquier *et al.*, 2012; Capone *et al.*, 2013; Schwarz and Johnson, 2016). The introduction of carbapenems, a class of broad-spectrum β-lactams also succumbed to the dissemination of hydrolysing enzymes including imipenamase (IMP), Verona integron-encoded MBL (VIM) and oxacillinase (OXA), New Delhi metallo-β-lactamase 1 (NDM-1) are being increasingly seen in Gram-negative bacteria (Bassetti *et al.*, 2011).

The global effort to develop new antibiotics or modify existing ones to fight resistant pathogens is a challenge. It is estimated that by 2050, the cost associated are predicted to increase to up to 100 trillion US dollars (O'Neill, 2016). New antimicrobials are needed but during the last three decades, antibiotic drug discovery has slowed and the pipeline against MDR Gram-negative bacteria is very limited and dominated by derivates of established classes. Recent developments are now focusing on natural products and the return of natural product screening to discover new therapeutics to fight resistance pathogens with ideally new mechanisms of action. β-lactamase inhibitors when administered with antibiotics can enhance the activity of the drug by blocking the resistance of the bacteria (Gill *et al.*, 2015). Currently, managing of *E. coli* bacteraemia may involve amoxicillin, piperacillin/tazobactam, gentamycin/gentamicin, ciprofloxacin or carbapenems (Hawkey *et al.*, 2018).

1.4 β-lactamases

In *Enterobacteriaceae*, there are two main mechanisms providing resistance to the much used β-lactam drugs namely target related including penicillin binding proteins and drug inactivation including all the β-lactamases.

1.4.1 Classification

Two schemes of classification were developed, one based on the amino acid sequences and an updated scheme based on the functional characteristics of the enzymes. The first classification consisted of four groups, classes A, B, C and D (Ambler *et al.*, 1991). Classes A, C, and D regroups hydrolytic enzymes with an active site serine, whereas class B β-lactamase are metallo-β-lactamases (MBLs). The current updated classification of β-lactamases groups these enzymes in three functional groups as shown in Table1.5 (Bush and Jacoby, 2010).

Table 1.5 Classification schemes for β-lactamases (Bassetti *et al.*, 2011; Ghafourian *et al.*, 2015).

Bush Group	Ambler class	Defining Characteristic(s)	Representative enzyme(s)
1	C	Not inhibited by clavulanic acid	AmpC, CMY-2
2a	A	Penicillinases	PC1
2b	A	Broad spectrum β-lactamases and mainly occurring in Gram-negatives	TEM-1, TEM-2 SHV-1
2be	A	ESBL Increased hydrolysis of oxyimino-β-lactams (cefotaxime, cephalosporins, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2c	A	Carbenicillin-hydrolysing enzymes	PSE-1, CARB-3
2d	D	Cloxacillin (oxacillin) hydrolysing enzymes	OXA-1, OXA-10
2e	D	Cephalosporinases inhibited by clavulanic acid	CepA
2f	D	Carbapenem-hydrolysing enzyme inhibited by clavulanic acid	KPC-2, IMI-1, SME-1
3	B	MBL that hydrolyse carbapenems and other β-lactams except monobactams. Not inhibited by clavulanic acid	IMP-1, VIM-1, CcrA, IND-1

Group 1 β-lactamases containing class C are active on cephalosporins and are usually resistant to inhibition by clavulanic acid. Those enzymes have activity on cefotaxime but not on ceftazidime (Yu *et al.*, 2008). Amino acid substitutions, insertions or deletions have led to the subgroup 1e which have a greater activity on ceftazidime and other oxyimino-β-lactams (Nordmann and Mammeri, 2007).

The group 2 includes the classes A and D and due to the increasing identifications of extended spectrum β-lactamase (ESBLs), this is the largest group of β-lactamases. The subgroup 2a penicillinases have a limited spectrum of hydrolytic activity. Subgroup 2b includes *bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-2}}$ and *bla*$_{\text{SHV}}$ which hydrolyse penicillins and early cephalosporins but are strongly inhibited by clavulanic acid and tazobactam. Further subgrouping exists including 2be comprising ESBLs having activity against penicillins, cephalosporins but also against oxyimino-β-lactams such as cefotaxime or ceftazidime and are sensitive to clavulanic acid. The group 2be has extended spectrum activity which is related to amino acid substitution in *bla*$_{\text{TEM-1}}$ which has given rise to a diversity of TEM-like enzymes with broadened substrate specificity. Similar but less extensive radiation occurred in *bla*$_{\text{SHV}}$. However members of the CYX-M enzymes are distinct and related to chromosomally borne β-lactamases in *Kluyvera* are part of this subgroup (Bonnet, 2004). Subgroup 2br are broad-spectrum β-lactamases that have acquired resistance to clavulanic acid, subgroup 2ber includes enzymes which have evolved some resistance to clavulanic acid. Subgroup 2c contains penicillinases that are easily inhibited by clavulanic acid and enzymes with the extended spectrum are included in 2ce. Subgroup 2d contains OXA-enzymes hydrolysing cloxacillin or oxacillin and extended spectrum enzymes including in 2de and the majority are derived from *bla*$_{\text{OXA-10}}$ and are able to degrade oxyimino-β-lactams. The subgroup 2df are OXA enzymes with carbapenem-hydrolysing activities. Resistance to extended spectrum cephalosporins and inhibition by clavulanic acid defined members of the group 2e and finally 2f are serine carbapenemases.

The group 3 contains the carbapenemases which are MBLs, require a zinc ion at the active site and are inhibited by metal chelators such as EDTA but not by clavulanic acid (Marchiaro *et al.*, 2008). In this group is found *bla*$_{\text{NDM-1}}$ which has become widely disseminated across a range of important bacterial pathogens including some of the members of the ESKAPE group (Yong *et al.*, 2009). Within group 3, there are three subgroups B1, B2, B3 based on the amino acids composition and a further three groups, 3a, 3b, 3c based on functional analysis (Bush and Jacoby, 2010). Subgroup 3a includes the major plasmid encoding MBL, *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$. 3b is the smallest group which will preferentially hydrolyse carbapenems compared to penicillin or cephalosporins.

1.4.2 CTX-M enzymes

Since 2000, *E. coli* producing CTX-M β-lactamases have emerged worldwide and now those enzymes are the most common type of ESBL enzymes in *Enterobacteriaceae* presenting challenges to healthcare with limited option to treat infections (Coque *et al.*, 2008a; Coque *et al.*, 2008b; Hawkey and Jones, 2009; Bush and Jacoby, 2010; Peirano and Pitout, 2010; Bevan *et al.*, 2017). Their global dissemination is due to the presence of *bla*$_{\text{CTX-M}}$ gene in highly mobilizable elements such as plasmids and transposons (Tn) but also because these elements are in successful pathogenic clones (Canton and Coque, 2006; Woodford *et al.*, 2009). Anthropogenic factors such as environmental pollution with animal and human waste, antibiotic misuse and human travel have all played a role in the selection and spread of these enzymes (Wellington *et al.*, 2013; Pehrsson *et al.*, 2016). More than 80 different enzymes have been identified and clustered into five groups based on their amino acid identities according to phylogenetic trees, the CTX-M-1, -2, -8, -9 and -25 groups (Poirel *et al.*, 2005).

CTX-M β-lactamases are encoded by genes that have been captured from the chromosome of *Kluyvera* species (Poirel *et al.*, 2008). The chromosomal *bla*$_{\text{kluC}}$ from *Kluyvera cryocresens* is considered as the ancestor of the *bla*$_{\text{CTX-M-1}}$ cluster of genes, the *bla*$_{\text{kluA}}$ from *K. ascorbate* is considered as the ancestor of the *bla*$_{\text{CTX-M-2}}$ gene cluster and the *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{CTX-M-15}}$ are believed to have originated from *K. georgiana bla*$_{\text{kluG}}$, *bla*$_{\text{kluY}}$ and *bla*$_{\text{CTX-M-78}}$ respectively (Decousser *et al.*, 2001; Humeniuk *et al.*, 2002; Poirel *et al.*, 2002; Rodriguez *et al.*, 2010; D'Andrea *et al.*, 2013) (Figure 1.4). The chromosomal *bla*$_{\text{klu}}$ genes are weakly expressed in their original context and required the presence of a strong promoter to be considered as phenotypically resistant. Insertion sequences (IS) elements have provided this promoter in cefotaxime-resistant *Enterobacteriaceae* strains, the most prevalent being ISEcp1 and ISCR1. ISEcp1 is frequently found upstream of *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ and played a role in the mobilisation of those ESBLs from *Kluyvera* species (Dhanji *et al.*, 2011b). The genes *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ are widespread globally with *bla*$_{\text{CTX-M-15}}$ the predominant variant in the UK and globally (Canton and Coque, 2006; Hawkey and Jones, 2009).

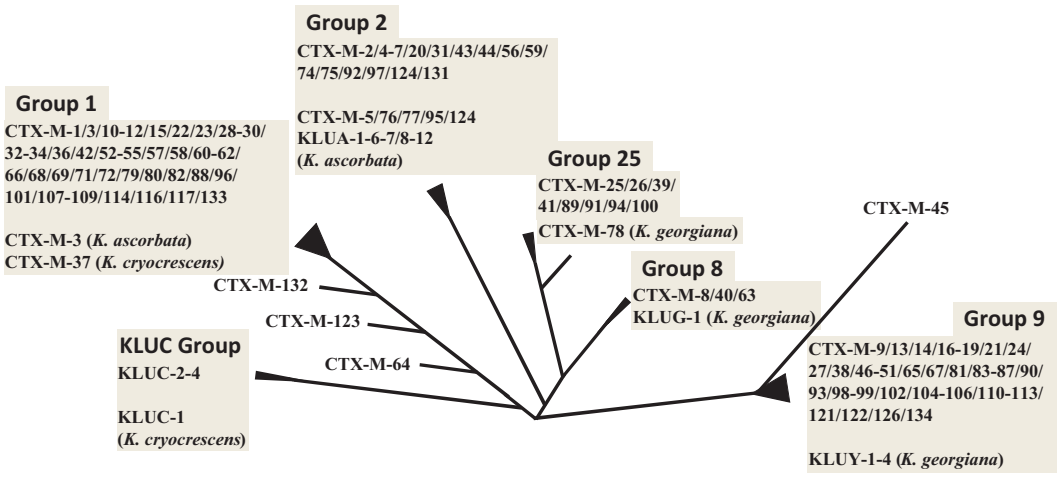


Figure 1.4 Tree showing the similarity among the CTX-M- lineage and clustering of the different group. The three was built with the available sequences of CTX-M and cognate proteins from *Kluyvera spp.* Figure is extracted from (D'Andrea *et al.*, 2013).

1.5 Mobile genetic elements (MGEs)

Questions still surround how indigenous resistance genes transfer to pathogens, how the selective pressures operate to drive this process and the role played by environmental transmission and environmental exposure (Amos *et al.*, 2014; Bengtsson-Palme *et al.*, 2018).

MGEs are platforms allowing capture, accumulation and dissemination of genes both intra or inter species and across phyla. A wide range of resistance genes have been mobilized by diverse MGEs and one of the main factor contributed to the development of ARB (Partridge *et al.*, 2018).

1.5.1 Plasmids in *Enterobacteriaceae*

Plasmids are defined as extrachromosomal elements of circular DNA which have the ability to replicate independently from the host genome, with mechanisms that allow them to capture, express and transfer genes within and between bacterial species. Plasmids contribute to the dissemination of antimicrobial resistance genes (ARGs) in a wide range of environments and the mobilization of ARGs has given rise to "superbugs" (Partridge *et al.*, 2009; Carattoli, 2013; Wellington *et al.*, 2013). Plasmids and integrons have been instrumental in the development of some of the most

important resistant pathogens within the ESKAPE group (Norman *et al.*, 2009). The normal average plasmid size can vary form 1 kb to 500 kb with a variation in the copy number per bacterial cell, large plasmids being limited to one to two copy per cell (Paulsson, 2002; Slater *et al.*, 2008) . Genes found on plasmids usually confer some benefits for host survival, such as ARGs, metal resistance, VFs allowing the host to be pathogenic or genes facilitating survival in other stressful conditions such as exposure to UVs and detergents (Datta and Richmond, 1966).

Conjugative plasmids are mobile and able to replicate and transfer to other bacterial species using the transfer (*tra*) region which encoded for genes required for the mating pair formation as well as a DNA transfer replication proteins (Figure 1.5) (Norman *et al.*, 2009). The mating pair formation function as a specialize type IV secretion system (T4SS) pore that will assembles a conjugative pilus in Gram-negative bacteria and allow contact with the neighbouring bacterium (Frost *et al.*, 2005; Smillie *et al.*, 2010). Conjugative plasmid can also encode for proteins called surface exclusion proteins that will prevent the host to acquire the same or related plasmid (Garcillan-Barcia and de la Cruz, 2008). Persistence of plasmids in a host population is based on four principles; efficient plasmid segregation during cell division, plasmid maintenance including plasmid-encoded toxin-antitoxin (TA) systems, conjugative transfer of plasmids to infect new cells and a low plasmid cost to avoid loss of an unfit host (Hayes, 2003; Ebersbach and Gerdes, 2005; Hall *et al.*, 2020).

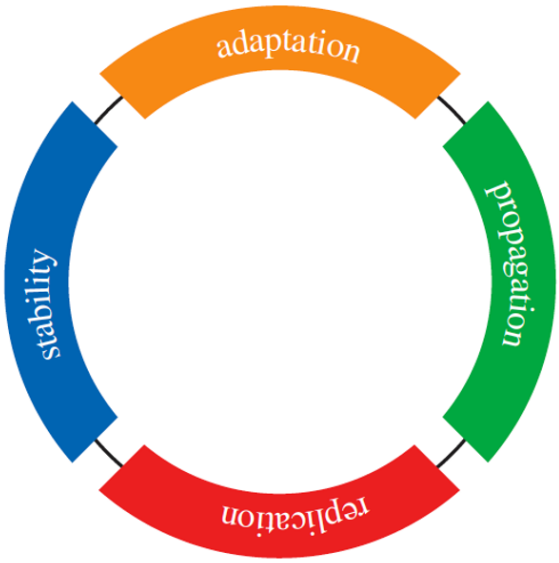


Figure 1.5 Organisation of a conjugative plasmid with the four genetic modules: stability (blue) where genes involved in partition systems are found , adaptation (orange) where the accessory genes are localised, propagation (green) which contain the *tra* genes important for the transfer and replication (red). Figure is extracted from (Norman *et al.*, 2009).

Plasmid classification has been defined by incompatibility (Inc) grouping since the 1970's and is determined by the amino acid sequences of the replication initiation (Rep) protein. Plasmids of the same ancestry are not compatible due to the same requirement for replication and partition (Novick, 1987; Shintani *et al.*, 2015). They can often carry more than one replicon making the classification into one Inc group difficult (Carattoli *et al.*, 2005; Shintani *et al.*, 2015). Twenty-seven Inc groups within *Enterobacteriaceae* have been identified and can be divided into narrow-host range corresponding to plasmids restricted to one specie and broad host range plasmid which can be transferred between different species (Table 1.6) (Carattoli, 2009; Carattoli, 2013; Shintani *et al.*, 2015). IncF plasmids are the most commonly detected in environmental and clinical isolates of *Enterobacteriaceae* and they often carry $bla_{\text{CTX-M-15}}$ in *E. coli* (Carattoli, 2009). Other $bla_{\text{CTX-M}}$ have been associated with IncN, IncI and IncL/M which contain other ARGs including *qnr* and *aac(6')-lb-cr* genes conferring resistance to fluoroquinolones and aminoglycosides respectively (Canton *et al.*, 2012). An alternative to the Inc classification is grouping plasmids according to mobility (MOB) type. This method is based on the relaxase gene which is usually carried in a single copy on a plasmid, therefore is only able to type transmissible plasmids (Table 1.6) (Garcillan-Barcia *et al.*, 2009; Garcillan-Barcia *et al.*, 2011). Six

groups are defined; MOB_C, MOB_F, MOB_H, MOB_P, MOB_Q and MOB_V (Smillie *et al.*, 2010).

Table 1.6 Summary of main characteristics of known resistance plasmids in *Enterobacteriaceae* (Partridge *et al.*, 2018; Rozwandowicz *et al.*, 2018).

Inc	Replicon(s)	Relaxase type[b]	Copy number[a]	Transferability	Host range
A/C	A/C	MOB_H	L	Conjugative	Broad
F	FII, FIA, FIB	MOB_F	L	Conjugative	*Enterobacteriaceae*
G	G	MOB_P	L	Mobilizable	Broad
HI1	HI1A, HI1B, FIA-like replicon	MOB_H	L	Conjugative	*Enterobacteriaceae*
HI2	HI1A, HI1B	MOB_H	L	Conjugative	*Enterobacteriaceae*
I	I1/B/O/K/Z	MOB_P	L	Conjugative	*Enterobacteriaceae*
L/M	L/M	MOB_P	L	Conjugative	Broad
N	N	MOB_F	L	Conjugative	Broad
P	P	MOB_P	L	Conjugative	Broad
Q	Q	MOB_Q	H	Mobilizable	Broad
R	R		L	Mobilizable	Broad
T	T	MOB_H	L	Conjugative	Narrow
U	U	MOB_P	L	Conjugative	Broad
W	W	MOB_F	L	Conjugative	Broad
X	X	MOB_P	L	Conjugative	*Enterobacteriaceae*
Y	Y	MOB_P	L	Plasmid-like prophage	*Enterobacteriaceae*
ColE1	ColE1	MOB_P	H	Mobilizable	

[a] H, high; l, low [b]MOB, mobility

1.5.2 Integrons, insertion sequences and transposons

Integrons allow the capture and expression of exogenous DNA in form of a gene cassette and they have played an important role in the spread of ARGs within Gram-negative bacteria (Gillings, 2014). They contain three key elements; an integrase gene (*IntI1*), a recombination site (*attI*) and a promoter (Pc) allowing the expression of genes present in the integron (Labbate *et al.*, 2009; Domingues *et al.*, 2012). The method of integration onto the chromosome of a host is achieved by site-specific recombination between the *attI* and *attC* sites and this allows efficient insertion and expression of a gene cassette on the integron. Mobilization can occur between chromosome and plasmids and between chromosomes and facilitating the spread of ARGs (Deng *et al.*, 2015). Over 130 different ARG cassettes have been identified on integrons providing resistance to most antibiotics used in treatment of Gram-negative

bacteria (Partridge *et al.*, 2009). Five classes of integrons based on the homology of the integrase proteins are known, with Classes 1, 2 and 3 with the Class 1 the most prevalent in the clinic and environment (Mazel, 2006; Deng *et al.*, 2015). Several studies have indicated a significant correlation between prevalence of *Intll* and ARGs in various population particularly in the environment, *IntI1* can act as a proxy for AMR by acting as a genetic platform for gene capture (Amos *et al.*, 2015; Gillings *et al.*, 2015). A sub-class of Class 1 was found in human-dominated ecosystems characterised by a 3' conserved regions, a truncated *qacEΔ1* biocide resistance gene and *sul1* sulfonamide gene (Figure 1.6) (Gillings *et al.*, 2015). These components are believed to have arisen through capture of an environmental betaproteobacterium integron containing *qacE* and subsequent *sul1* sulfonamide resistance gene which led to a truncated *qacE* (Stokes and Hall, 1989; Paulsen *et al.*, 1993; Skold, 2000).

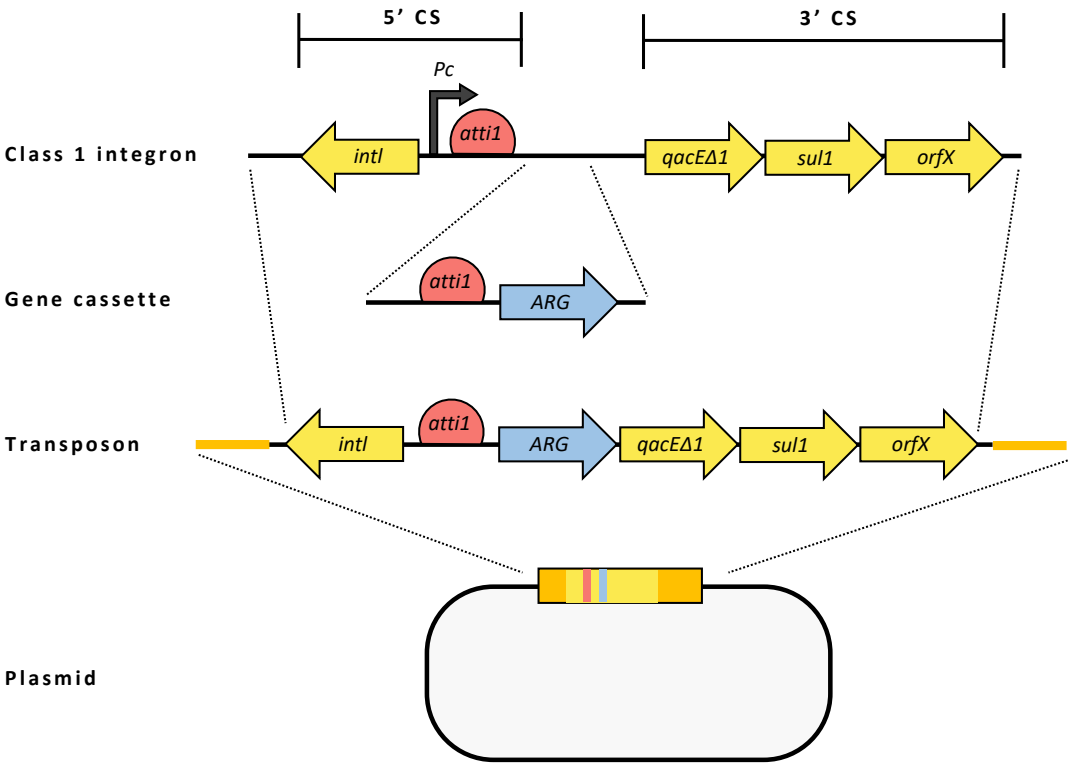


Figure 1.6 Structure of Class 1 integrons captured by a transposon and inserted into a plasmid The integron consist of the integrate site (*IntI1*), the recombination site (*attI*) and the promotor (Pc).

Insertion sequences (IS) and transposons (Tns) are a type of MGE, these are able to rearrange chromosomal DNA and can move from cell to cell through plasmids or phage and integrative conjugative elements (Frost *et al.*, 2005; Partridge *et al.*, 2018). *E. coli* in particular contains a high number of IS, these can cause abnormalities and mutations within the genome, this generally occurs when they transpose to the middle of a gene sequence. IS are between 0.8 and 2.5 kb and will contain a transposase (*tnp)* gene required for transposition which can occurr by a copy-and-paste or a copy-out-and-paste-in mechanism (Hallet and Sherratt, 1997; Chandler *et al.*, 2015). They are usually bounded by inverted repeat region of ~40 bp which are specific for each IS and may carry partial or complete strong promoters which activate the expression of the captured genes (Mahillon, 1998). IS are able to move resistance genes as part of a composite Tn which is defined as a region bounded by two IS elements (Figure 1.7). It has been suggested that IS played a role in the expression of resistance to β-lactams, aminoglycosides, quinolones, glycopeptide, imidazoles and tetracyclines (Depardieu *et al.*, 2007).

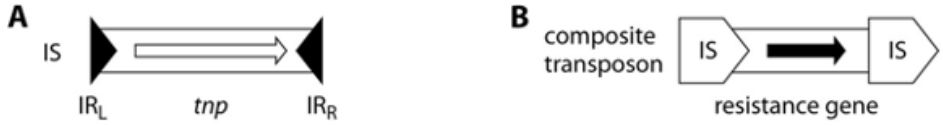


Figure 1.7 Insertions sequences (IS) and composite transposons. **(A)** Structure of IS. **(B)** Structure of a composite transposon. The black arrow represents the resistance gene. IR_L: left inverted repeat; IR_R: right inverted repeat, *tnp*: transposase gene. Figure is adapted from (Partridge *et al.*, 2018).

1.6 *E. coli* ST131

1.6.1 Dissemination and impact of the ExPEC *E. coli* ST131

Amongst different STs and strains of *E. coli*, the ExPEC *E. coli* ST131 has emerged as a major healthcare problem due to its prevalence in the community as an asymptomatic member of the gut microbiome and frequent occurrence as UTIs. This has resulted in dissemination of ST131 to the wider environment such as WWTPs and rivers (Zurfluh *et al.*, 2013; Amos *et al.*, 2014; Nicolas-Chanoine *et al.*, 2014). Initially, ST131 was identified in a limited number of countries before being disseminated worldwide (Figure 1.8). Its success is related to its ability to persist in the mammalian gut (Gibreel *et al.*, 2012). The association of different virulence determinants encoding for toxins, adhesins and siderophores with MDR may be the reason for the ecological success of this clone (Nicolas-Chanoine *et al.*, 2014). The presence of VFs such as the secreted autotransporter toxin (*sat*), the aerobactin receptor (*iutA*) and the outer membrane protein (*ompT*) are specific to ST131 and may play a part in its fitness and adaptation by increasing its ability to efficiently colonize human tissues and the gut (Pitout, 2012; Whitmer *et al.*, 2019). As a member of the ExPEC group, ST131 can cause a wide spectrum of infections contributing to an increase in the degree of concern about this strain which is already considered a major problem due to its multidrug resistance.

Figure 1.8 Global dissemination of *E. coli* ST131 (2013). Red stars indicate isolates producing ESBL enzymes and blue stars indicate fluoroquinolone-resistant, non-ESBL-producing isolates. Figure is extracted from (Nicolas-Chanoine *et al.*, 2014).

1.6.2 Antimicrobial resistance genes (ARGs) and plasmids

ST131 initially acquired β-lactam resistance and further gene transfer events, mainly plasmid related, resulted in acquisition of fluoroquinolone-resistant (Nicolas-Chanoine *et al.*, 2014). The worldwide appearance of *E. coli* with $bla_{\text{CTX-M-15}}$ was due to the acquisition of IncF plasmid harbouring the $bla_{\text{CTX-M-15}}$ in ST131, but it can harbour other ESBLs such as $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-M-12}}$. Presence of β-lactamases genes limits treatment options and increases the use of last-resort antimicrobials such as carbapenems and colistin (Johnson *et al.*, 2010). A surveillance study showed that ST131 is responsible for 60% to 80% of fluoroquinolone-resistant isolates, 50% to 60% of ESBL-producing isolates but only up to 7% of fluoroquinolone-susceptible isolates (Banerjee and Johnson, 2014). Other resistance determinants that have been characterized in isolates include *mph(A)* (resistance to the macrolides), *catB4* (resistance to chloramphenicol), *tetA* (resistance to tetracycline resistance), *dfrA7* (resistance to trimethoprim), *aadA5* (resistance to streptomycin), and *sul1* (resistance to sulphonamide) and the presence of plasmid mediated quinolone resistance determinant *aac(6)-lb-cr* (Peirano and Pitout, 2010; Nicolas-Chanoine *et al.*, 2014; Mathers *et al.*, 2015). Acquisition of plasmids and resistance genes are generally associated with fitness cost, however, ESBL-producing ST131 strains do not seem to

have a fitness disadvantage. This can be explained by the presence of genes important for host survival and the possible interaction and regulation of chromosomally encoded pathogenic factors by plasmids harboured by ESBL-producing ST131 (Schaufler *et al.*, 2016).

1.6.3 Environmental non-host associated occurrence of *E. coli* ST131

The intestinal carriage of ESBL-producing *E. coli* is frequent in patients in both hospital and community settings. The digestive tracts of livestock also constitute a large reservoir of these bacteria. *E. coli* are released into the environment through faeces either directly or after treatment at WWTPs (Figure 1.9).

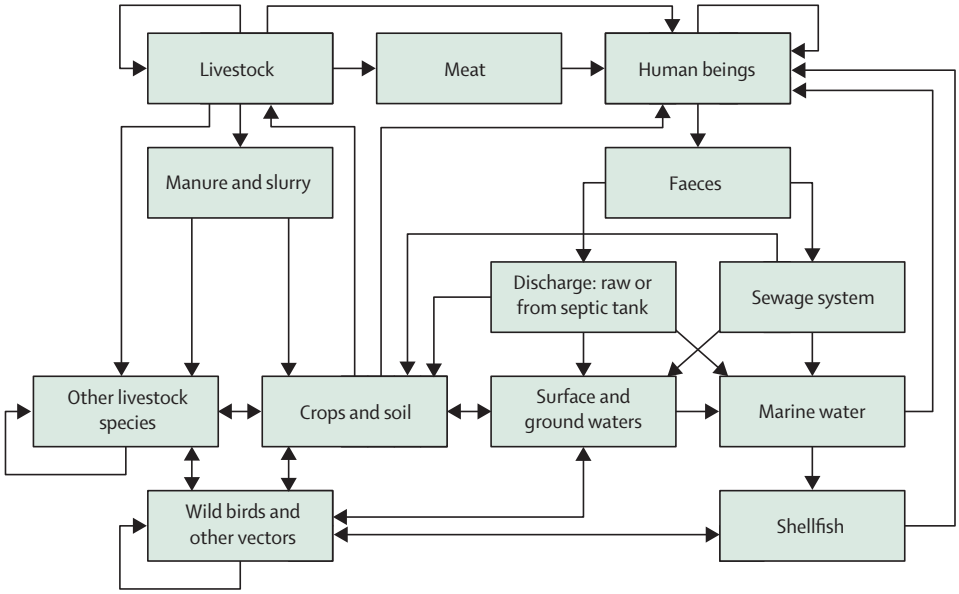


Figure 1.9 The role of the environment in the dissemination of ARGs and ARBs. Figure is extracted from (Wellington *et al.*, 2013).

WWTPs process waste from numerous sources from hospitals, communities, industrial settings as well as greywater and the mixing of bacteria from multiple sources generates a hotspot for horizontal gene transfer (HGT) between bacteria (Volkmann *et al.*, 2004; Rizzo *et al.*, 2013). The characteristic of the influent is highly variable, depending on the catchment characteristics, the presence of hospital and the type of plant (secondary or tertiary) and its operational parameters (mesophilic or thermophilic.). Furthermore, the colocation of antibiotics and ARGs in WWTPs can select for novel combinations (Singer *et al.*, 2016). Different types of antibiotics present in wastewater could play a role in the selection of resistance and contribute to

resistance dissemination and retention. In the sewage treatment, there are two end products: the treated effluent goes directly into the rivers and the digested sludge is put onto land (Figure 1.10).

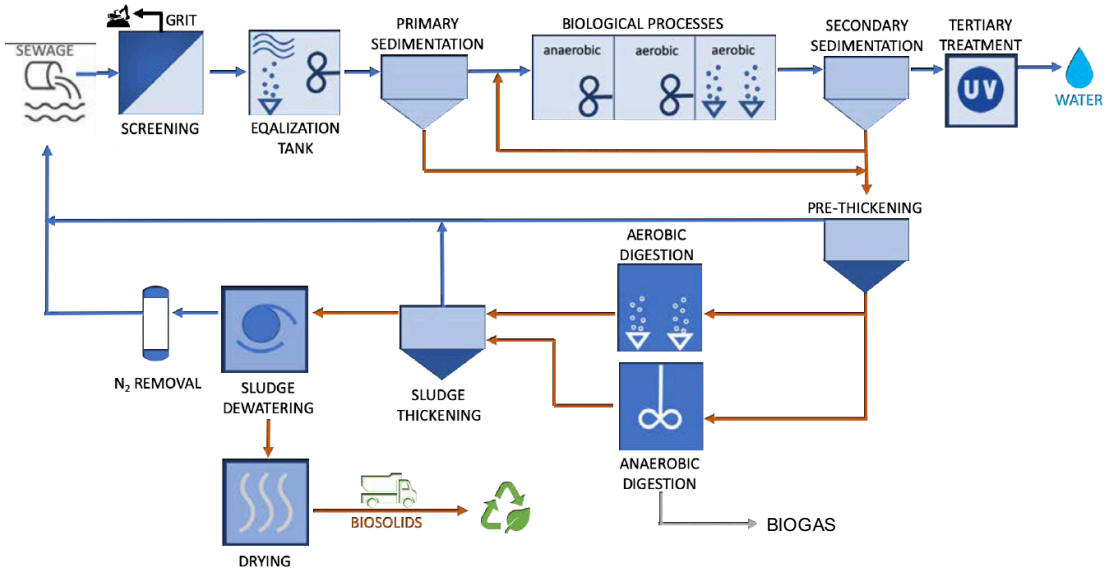


Figure 1.10 Diagram of the main stages involved in the WWTP process. Figure is adapted from (Jazbec and Turner, 2020).

Rivers impacted by WWTP effluent represent one of the most extreme examples of human pollution of the environment (Martinez, 2009). Nevertheless, data on the prevalence of ESBL-producing *E. coli* in hospital and community environments and in effluent from WWTPs are limited (Mesa *et al.*, 2006; Jakobsen *et al.*, 2008; Galvin *et al.*, 2010). However, it has been shown that pathogenic *E. coli* may have an advantage over commensal strains in the anaerobic digesters of WWTPs due to the selective pressure exerted by antibiotics (Galvin *et al.*, 2010). Haberecht *et al.* (2019) showed a decrease of *E. coli* abundance after WWTPs processing but an increase of the relative abundance (1.7%) of ESBL resistance bacteria in surface water (Haberecht *et al.*, 2019). In line with this study, ESBL-producing *E. coli* can therefore enter the environment from the WWTPs effluent released into the river (Amos *et al.*, 2014). After settling, the sludge from the anaerobic tank is disposed to land and used as fertilizer which also will contain amount of *E. coli*. Galvin *et al.* (2010) estimated number of *E. coli* isolate (as most probable number/100 ml) in the treated effluent varied from 5.76 x 10^3 to 1.55 x 10^5 (Galvin *et al.*, 2010). ST131 isolates have been detected in non-human sources such as companion animals, livestock, food chain and

the environment but due to the limited data available, it is impossible to draw a definitive conclusion about the amount of *E. coli* ST131 (with or without ESBL production) released into the environment and whether it can grow there (Arpin *et al.*, 2009).

Research aims for the Ph.D. thesis

Previous work done in the River Sowe, downstream of a WWTP identified and isolated pathogenic clones of ST131 as well as other strains (Hill, 2016). The strains were prevalent in the river and subsequent study showed that they were able to grow between 10°C and 40°C. The factors influencing the survival and the dissemination of *Enterobacteriaceae* and in particular ExPEC *E. coli* ST131 have not been intensively studied yet. Further work was needed to fully characterise these important pathogens surviving in the environment, establish their relatedness to other STs and understand the mechanism they possess for antibiotics resistance. In particular, the characterization of one *E. coli* ST131 isolate, strain 48, showing the presence of *bla*$_{\text{CTX-M-15}}$ gene in the WGS previously performed was of interest for this study (Hill, 2016).

Both *in-vivo* and *in-vitro* studies have demonstrated that β-lactamase producing bacteria exert a protective effect nearby susceptible strains by releasing the enzyme into the immediate environment. However, the mechanism of β-lactamase secretion in *E. coli* remains elusive. In our study, we aimed to understand the mechanisms of enzyme secretion of the CTX-M-15 in the *E. coli* strain 48, determining the secretory pathway involved and whether a protective effect was provided in the presence of antibiotic.

The key aims of the project can be summarised as follow:

Aim 1: Further characterisation of the ST131 genome of strain 48, its phylogeny and detailed analysis of the genetic contexts of the resistance genes, particularly *bla*$_{\text{CTX-M-15}}$.

Objective 1: Characterise the plasmids harboured by the *E. coli* ST131 namely 48 isolated downstream of a WWTP.

Hypothesis 1: Strain 48 carries multiple plasmids encoding for resistance genes and VFs.

Aim 2: Gain a better understanding of the CTX-M-15 enzyme and its cellular location.

Objective 2: Investigate the evidence that CTX-M-15 is secreted, its mechanism of secretion which could explain the success of this enzyme and characterisation of the *bla*$_{\text{CTX-M-15}}$ gene expression.
Hypothesis 2: CTX-M-15 is secreted and provide protection to surrounding cells.

Aim 3: Better understand the fitness of ST131 strain 48 and considered the ability to survive anaerobic digestion given the original location of that strain, downstream of a WWTP.
Objective 3: setting-up of small-scale anaerobic bioreactors (AB) and compare survival of non-resistant commensal bacteria with *E. coli* ST131.
Hypothesis 3: AB reduces the load of bacteria and resistant *E. coli* survive better than the non-resistant *E. coli*.

Chapter 2:

Genetic location and characteristics of resistance genes in *E coli* 48.

2.1 Introduction and aims

ARGs are often located on MGEs such as transposons (Tns), integrons, integrative conjugative elements (ICEs) and plasmids, all with varying degrees of mobility between community members. For example, ARGs can form cassette genes which can be readily mobilised in integrons, such as a Class 1 integron, commonly found on conjugative plasmids (Gillings *et al.*, 2008). ARGs can be flanked by IS and mobilised to a plasmid or chromosomal location using the transposase gene of the IS element. Genes can also be mobilised by an IS element as part of a composite Tn.

The persistence of ARGs outside of the host-associated system may play a role in shaping the community-acquired resistome through HGT (Partridge *et al.*, 2018). Plasmids are known to facilitate the transfer of ARGs between bacteria from distant taxonomic lineages through both HGT and the acquisition of IS and Tns (Thomas and Nielsen, 2005; Popowska and Krawczyk-Balska, 2013). Such plasmids can carry VFs allowing their stability and maintenance in a bacterial host and often can confer resistance to different classes of antibiotics (Carattoli, 2009; Carattoli, 2013). The identification and characterisation of plasmids provides information concerning the transmission of genes and will help to identify the mechanisms contributing to the spread of ARGs among bacteria (Rozwandowicz *et al.*, 2018).

Resolving plasmid structure from WGS using short-read data is challenging as plasmids contain repetitive sequences, some of which they may share with the chromosome (Schatz *et al.*, 2010; de Toro *et al.*, 2014; Arredondo-Alonso *et al.*, 2017). Illumina paired end sequencing technologies produce short reads of 50-300 bp, thus the identification and resolution of repetitive IS within both the plasmid and the genome cannot be performed with any accuracy or precision (Ashton *et al.*, 2015). Multiple plasmids can also co-habit within the same host which share conserved

regions of genetic homology, therefore assembling and resolving the complete closed sequence of individual plasmids can be problematic. The limited ability to resolve the genetic context of plasmid borne AMR genes has thus far limited our understanding into the persistence, spread and evolution of antibiotic resistance in environmental communities.

Long-read sequencing technologies such as Pacific Biosciences (PacBio) Single-Molecule Real-time and MinION sequencing from Oxford Nanopore Technologies (ONT) can overcome the issues of plasmid assembly due to the ability to span multiple repeat regions and areas of shared homology. PacBio offers high read accuracy through sequencing by synthesis but remains a costly technology with limited raw read length from an average of 10 kb and maximum over 60 kb (Conlan *et al.*, 2016). The MinION from ONT is a small and rapid long-read sequencer that sequences native DNA directly through measurement of current fluctuations as DNA is passed through a biological pore (Nanopore, 2016). The technology offers read lengths that can reach a maximum of 2,500 kb, but this has a major drawback of a maximum raw read accuracy of 95%. These errors can be corrected by "polishing", a technique used to identify and remove errors through raw read consensus, and by the use of Illumina data to perform a hybrid / polishing assembly. This method combines high accurate data from the Illumina platform (read accuracy of 99.9%) with the long-read data from the MinION platform (Jain *et al.*, 2015; Laver *et al.*, 2015). The long reads allow identification of gene order and position of repeat regions such Tns and ISs. Thus, providing information in relation to location of specific genes, while the short reads are used to correct indels (single base pair errors) within the complete consensus sequence.

Previous work undertaken in the Wellington group isolated a large number of *E. coli* downstream of a WWTP from the River Sowe (UK) (Amos *et al.*, 2014; Amos *et al.*, 2018). WGS provided data on the presence of a high diversity of ARGs within these isolates. The *E. coli* ST131 isolate named "48" was selected from this strain bank for further characterisation based on the presence of multiple ESBL genes of clinical relevance (Table 2.1). The aim of this study was to develop a method to rapidly sequence and produce a highly contiguous whole genome assembly for strain 48 in order to resolve the genetic location and context of the $bla_{\text{CTX-M-15}}$, bla_{TEM} and bla_{OXA}.

We hypothesised that these three β-lactamase genes are carried on conjugative plasmids within this environmental isolate. Understanding the ARG location in essential for future work on the study of the fate of *E. coli* 48 in anaerobic digesters and the stability of the resistance phenotype in liquid culture.

2.2 Material and methods

2.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 2.1. Environmental *E. coli* ST131 O25:H4 namely strain 48 was isolated from the River Sowe (Hill, 2016). *E. coli* ET 12567 was acquired for conjugation studies.

Cells were routinely grown in Luria Bertani (LB) liquid broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or LB solid (addition of 15 g/L agar) medium, supplemented with 8 μg/ml of cefotaxime, 25 μg/ml of chloramphenicol. Cells were incubated at 37ºC with either shaking (200 rpm) or static conditions.

All *E. coli* strains were stored in 8% (v/v) glycerol at -80ºC.

Table 2.1 Bacterial strains and plasmids used in this study.

Strain	Genotype and comments	Reference
E. coli 48	$bla_{CTX\text{-}M\text{-}15}$, bla_{TEM}, bla_{OXA}, *sul1, vanG*	This study
E. coli ET12567	*Dam-13:: Tn9 dcm-6 hsM* Chl^R	ATCC

2.2.2 Primers for cloning and screening

Specific primers for amplification of resistance genes in this study were designed from the Illumina sequencing done previously (Hill, 2016) (Table 2.2). PCR reactions were done using 12.5 μl Master Mix 2X (Promega), 1.25 μl DMSO, 0.8 μM forward primer, 0.8 μM reverse primer, 2 μl DNA template and deionized water (dH_2O) for a final PCR reaction volume of 25 μl. PCR was performed at an initial denaturation temperature at 95ºC for 5 min, followed by 34 cycles of denaturation at 95ºC for 30 sec, annealing temperature (Ta) at 55-66ºC (depending on the primer set) for 30 sec and extension for 1 min 50 sec. A final extension was performed at 72ºC for 5 min.

Table 2.2 Details of primers pairs used in this study for PCR screening.

Target	ID	Sequence	Ta (°C)	Size (bp)	Reference
bla$_{CTX-M\ 15}$	CTX-M_F	5'ATGGT AAAAA TCACT CGCCA GTTC 3'	63	876	This study
	CTX-M_R	5'TTACA AACCG TCGGT GACGA T 3'			
bla$_{TEM}$	TEM_F	5'ATGCG CCTGG TAAGC AGAGT 3'	55	1124	This study
	TEM_R	5'TTACC AATGC TTAAT CAGTG 3'			
bla$_{OXA}$	OXA_F	5'CAGTT ACTGG CGAAT GCATC 3'	66	1287	This study
	OXA_R	5'CGTCC CGACT TGATT GAAG 3'			
cat	Cm_F	5'ACCGT AACAC GCCAC ATCTT 3'	55	471	This study
	Cm_R	5'TTCTT GCCCG CCTGA TGAAT 3'			

2.2.3 Conjugation

E. coli ET12567 (recipient) and strain 48 (donor) were first grown individually in LB with 25 μg/ml of chloramphenicol and 8 μg/ml of cefotaxime respectively. Cultures were incubated overnight (o/n) at 37°C in a shaking incubator at 200 rpm. 200 μl of the two o/n cultures was individually inoculated into 10 ml of LB containing 25 μg/ml and 8 μg/ml of chloramphenicol and cefotaxime respectively. Cells were harvested at an A_{600} nm of 0.4-0.6 and centrifugated at 2,000 rpm for 5 min at room temperature. The pellet was gently re-suspended into 500 μl of fresh LB. This step was repeated twice, and the pellet was then suspended into 1 ml of LB. 20 μl of each strain was spotted onto the same location on a LB agar plate containing no antibiotic and was incubated o/n at 37°C. The o/n incubated spot was streaked onto a LB agar plate containing 8 μg/ml cefotaxime and 25 μg/ml of chloramphenicol and was incubated o/n at 37°C to select for transconjugants (TC). TC were checked by PCR for the presence of the *bla*$_{CTX-M-15}$ and the chloramphenicol gene (*cat*) (primers in Table 2.2).

2.2.4 DNA extraction

2.2.4.1 TC: plasmid isolation

Plasmid DNA was extracted from TC using the BAXMAC™ DNA (Epicentre) purification kit following the manufacturer guidelines. Aliquot of 10 ml o/n culture were used for the extraction.

2.2.4.2 *E. coli* 48 DNA

Genomic and plasmid DNA was extracted from strain 48 using the Qiagen genomic tip 500 kit and manufacturer-based protocol from 15 ml o/n culture supplemented with 8 μg/ml of cefotaxime. DNA was eluted in elution buffer at 56^{o}C and precipitated with 100 % isopropanol. DNA was spooled onto a closed end glass Pasteur pipette and immediately placed in 1 ml of 1x TE then the DNA was dissolved at 4^{o}C on a rotating mixer for 5 days.

2.2.4.3 DNA quantification

DNA concentration was quantified using the Qubit fluorometer (Invitrogen). The Qubit dsDNA Broad Range BR kit (Invitrogen) was used according to the manufacturer guidelines. The purity of the DNA samples was assessed using the Spectrophotometer NanoDrop^{TM} 2000. A ratio A_{260}/A_{280} ~1.8 is accepted as "pure" for DNA, a lower ratio may indicate the presence of protein, phenol or other contaminants. The ration A_{260}/A_{230} is used as secondary measure to assess the purity of nucleic acid; values are expected to be in the range of 2.0-2.2. Values lower than expected may indicate the presence of contaminants.

2.2.5 MinION library preparation

Following DNA extraction, the Oxford Nanopore ligation kit (LSK109) was used to prepare the sequencing reactions following the 1D Native barcoding genomic DNA protocol. In order to limit the amount of shearing of the DNA, pipetting was done with care.

2.2.5.1 DNA fragmentation

1.5 µg of DNA was mechanically sheared using a g-TUBE™ (Covaris) following manufacturer guidelines. Centrifugations were done at 4,200 rpm using an Eppendorf® 5424 microfuge.

2.2.5.2 DNA repair and end-prep repair

DNA Repair Mix (FFPE™ New England BioLabs) was used for the Formalin-Fixed, Paraffin-Embedded (FFPE) repair. End-repair and dA-tailing were performed using the NEBNext Ultra II End-repair Mix/dA-tailing Module (New England BioLabs) following the SQK-LSK 109 with modification as follows; incubation at 20°C and 65°C were completed for 15 min, and the final incubation with Nuclease-free water was performed for 30 min at 37°C. A 0.5 x Solid Phase Reversible Immobilisation (SPRI) beads (Beckman Coulter) clean-up was undertaken and subsequent DNA was quantified.

2.2.5.3 Barcode ligation

Barcodes of known nucleotide sequences were ligated to each end-prepped DNA sample, originating from the plasmid isolate and whole-genome sample to permit multiplexing of samples. Barcodes were ligated using blunt TA-master mix (New England BioLabs) for 15 min at room temperature. A 0.5 x SPRI clean-up was undertaken and DNA was quantified.

2.2.5.4 Adapter ligation

Ligation was performed using the T4 Quick Ligase kit (New England BioLabs) and the adapter Mix (ONT) and incubation was performed at 37°C for 15 min. A manufacturer modified 0.5 x SPRI clean-up was undertaken using the Long Fragment Buffer supplied with the sequencing kit. DNA was eluted for 20 min at 37°C in 12 µL of manufacturer supplied elution buffer and retained for flow cell loading.

2.2.5.5 Sequencing

Two sequencing reactions were undertaken in this study. The first sequencing run was performed to sequence the isolated plasmid containing the *bla*$_{\text{CTX-M-15}}$ gene and the latter to undertake WGS on the isolate and all associated plasmids. Both sequencing reactions were performed on an ONT MinION device with a final library concentration of 200 ng. The sequencing reactions were left to progress for 5 h before concluding. The reaction was run on R9.4.1 flow cells (FLO-MIN 106) and was controlled with the desktop Graphic User Interface v2.0 (GUI) and the MinKNOW v1. Fast5 files were retained and uploaded to the CLIMB servers (Connor *et al.*, 2016) for onward basecalling and assembly.

A previously existing short-read data set was also used in this study (Hill, 2016). Sequencing was undertaken on an Illumina Miseq using the Illumina Nextera MiSeq reagent kit 2 x 150 bp.

2.2.5.6 Basecalling and Assembly

Raw reads were basecalled using Guppy v3.4.1 with the high accuracy (HAC) config file. Barcodes were demultiplexed using Guppy and then qCat v1.1.0, and Filtlong v0.2.0 was used to filter the basecalled reads with the quality filter bias set to 18 and the minimum read length cut off set at 1.2 kb. A *de novo* assembly was first performed using the program Flye 2.7b with the plasmid flag enabled to ensure small circular contig retention (Kolmogorov *et al.*, 2018). Once a genome is assembled, assembly errors can remain in the assembly in the form of additions, deletions and substitutions (indels) which lead to frameshifts and result in premature or extended stop codons in a protein-coding region. Racon and Pilon were used to correct the assemblies using a consensus orientated approach for long and short reads respectively; the assembled contigs were polished using four rounds of Racon v1.4.3 polishing (Vaser *et al.*, 2017) and passed to Medaka v0.11.5 to complete the long-read assembly. Following this, four rounds of Pilon (Walker *et al.*, 2014) polishing were undertaken. Indel was used to assess sequence contiguity by identifying the presence of indel sequencing errors leading to frame shifts and ORF truncation. Predicted proteins were searched against

the TrembleUniprot protein database, and the length of the predicted protein was compared with the length of the top hit. The frequency of length ratio between queries and hits are used as an indicator of indel frequency.

2.2.5.7 Gene annotation

A custom database of *E. coli* ST131 with annotated plasmid sequences was constructed using the NCBI database for comparison with 243 fully annotated plasmids associated with both *E. coli* ST131 and ESBLs. Prokka v1.14.5 was used with the proteins flag and the custom database firstly to identify the open reading frame (ORF) and secondly to annotate the identified ORFs from the reference database (Seemann, 2014). The assembled contigs were also queried against the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca) with a minimum identity threshold of 80 % to validate the Prokka analysis. PlasmidFinder 2.1 (Carattoli *et al.*, 2014) was used to identify the plasmid incompatibly group (Inc group) and the origin of transfer (oriT) with a minimum identity threshold of 80% and coverage of 80%. Plasmid maps were constructed using Artemis v18.0.1 and DNA viewer (Carver *et al.*, 2012) (Figure 2.1).

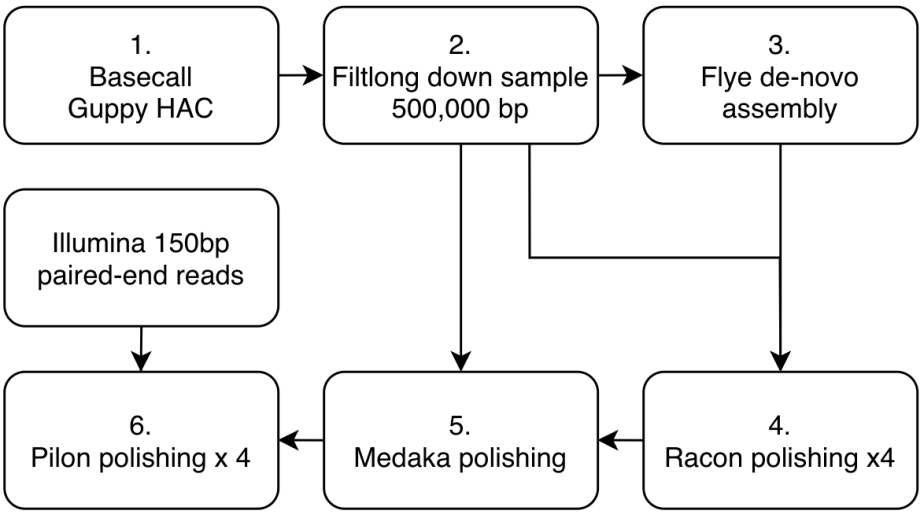


Figure 2.1 Bioinformatic pipeline used for basecalling, assembly and annotation.

2.3 Results

2.3.1 Verification of the DNA extraction

2.3.1.1 DNA extracted from the TC

Colony PCR confirmed the presence of the *bla*$_{\text{CTX-M-15}}$ and the chloramphenicol gene *cat* in the TC. Transfer with selection was preferred before proceeding to sequencing in order to lower the possibility of having two or more plasmids in the recipient cells.

Plasmid DNA was successfully extracted from the TC and PCR indicated the presence of one ESBLs gene, the *bla*$_{\text{CTX-M-15}}$ in the plasmid extract.

2.3.1.2 DNA extracted form strain 48

An agarose gel was run in order to control and visualize the extraction. Lane E was loaded with the extraction, the upper band very close to the well represented the high molecular weight genomic DNA, followed by the supercoiled form of the plasmid (Figure 2.2).

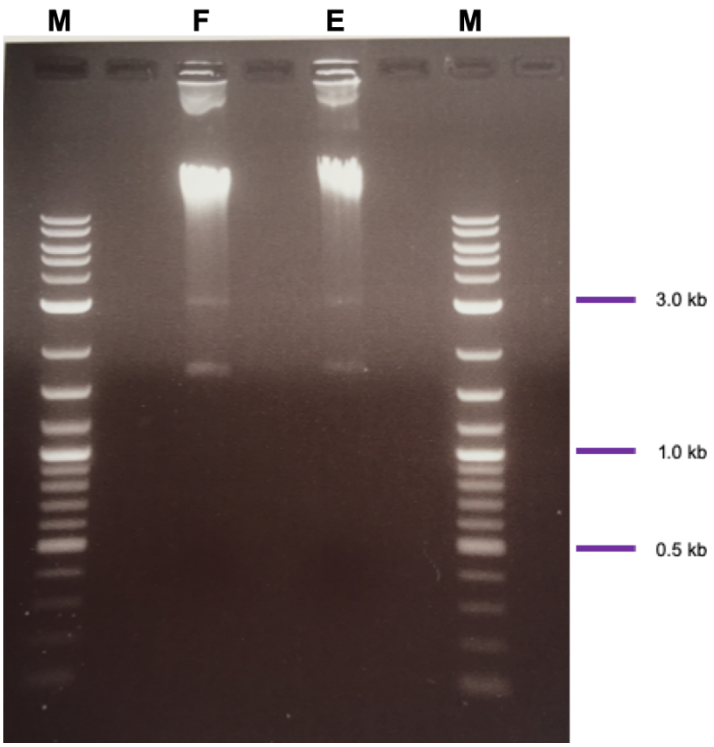


Figure 2.2 DNA extracted from strain 48 visualized on a 1% (w/v) agarose gel (TAE 1X buffer). M= 1kb ladder plus (New England BioLabs), F= fragmented extract, E=Extraction using Qiagen genomic tip 500.

PCR using the *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$ primers showed the presence of the three genes in the DNA extracted from strain 48 (Figure 2.3).

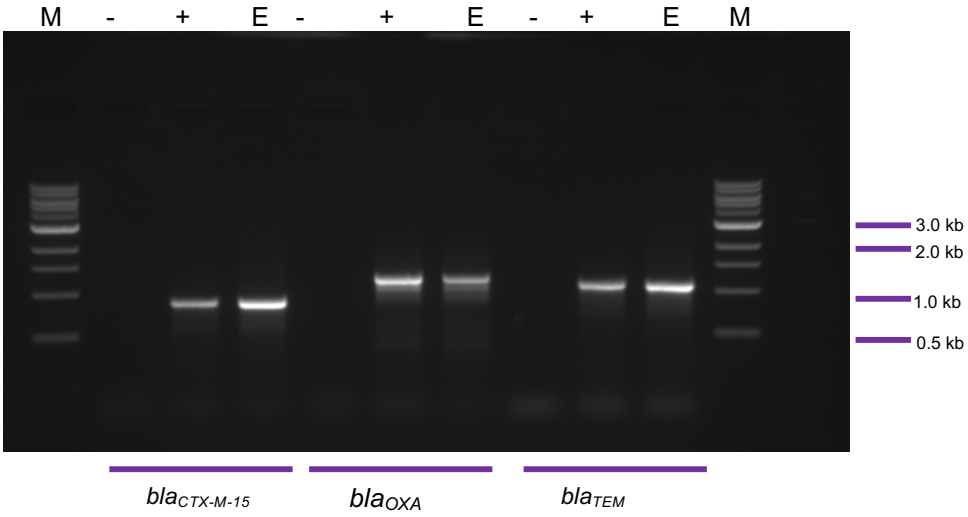


Figure 2.3 1% (w/v) agarose gel showing PCR products from DNA extracted from strain 48 using CTX-M_F/ primers, TEM_F/R primers, OXA_F/R pimers. M= 1kb ladder (New England BioLabs), - = dH_2O, += DNA from *E. coli* 48 using the FastPrep DNA kit, E=Extraction using the Qiagen genomic tip 500.

2.3.1.3 DNA concentration

The concentration and the purity of the DNA obtained after extractions are shown in Table 2.3.

Table 2.3 Purity and DNA concentration of TC and 48 extracts.

Sample	A260/280	A260/230	Concentration (ng/µl)	Concentration (µg)
TC	1.78	1.42	41.90	1.04
48	1.81	1.39	99.20	49.60

2.3.2 Library preparation

2.3.2.1 Fragmentation: Covaris g-TUBE™

Prior to sequencing, DNA extracts were mechanically fragmented with a maximum read length of 52 kb and 196 kb for the TC and 48 respectively. And average length of 10 kb and 16 kb was reached for the TC and 48 respectively (Figure 2.4).

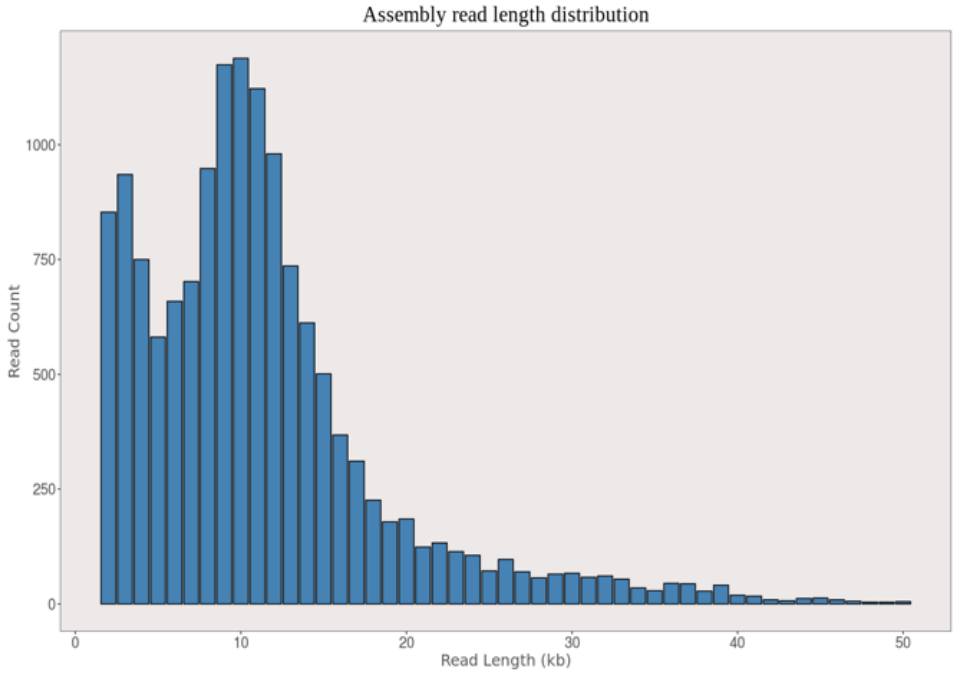


Figure 2.4 Read length distribution used for the plasmid assembly following DNA fragmentation of the TC with the Covaris g-TUBE™.

2.3.2.2 MinION sequencing run

After the fragmentation, libraries were prepared and sequenced with the MinION sequencer. Two sequencing reactions were performed, one using the plasmid containing the *bla*$_{\text{CTX-M-15}}$ and the second one was the WGS of strain 48. The two runs were left for 5 h, the one with TC generated 2.1 Gb of sequence data with a depth coverage of 337x. The second one, for the WGS generated 52,000 reads with a mean coverage of 406x (Table 2.4). Three contigs were obtained for the WGS assembly. One contig corresponded to the genome and the remaining two corresponding to two independent plasmids.

Table 2.4 Assembly statistics for TC and 48.

Extraction	TC	48
Reads	15,000	52,000
Average read length (Kb)	10.2	17.6
N50 (bp)	10,153	17,610
Coverage	337x	406x
Unique unitigs	5620	0

2.3.3 Annotation

2.3.3.1 Inc group

bla$_{\text{CTX-M-15}}$ was identified on an IncB/O/K/Z plasmid with 97 % identity. *bla*$_{\text{TEM}}$ was identified on an IncFIB/IncFII plasmid in association with other ARGs; *aph(6)-id; aph(3")-lb*; *sul2* and *dfrA17* (Table 2.5)

Table 2.5 Features of the plasmids.

Plasmid	Replicon	ARGs
pSRJ48c	IncB/O/K/Z	*bla*$_{\text{CTX-M-15}}$
pSRJ48t	IncFIB/IncFII	*bla*$_{\text{TEM}}$; *aph(6)-id; aph(3")-lb*; *sul2*; *dfrA17*

2.3.3.2 Indel analysis

After the four rounds of Pilon polishing, the assemblies for both the TC and the WGS assemblies were evaluated using indel plots. The indel analysis showed that >95 % of called ORFs were of equal length to their corresponding top hit in the uniport Tremble protein database with a length ration of 1:1. Approximately 4% of called ORFs were

shorter than the full length of their corresponding top hit in the database and 1% were longer (Figure 2.5). For the plasmid carrying the *bla*$_{\text{CTX-M-15}}$, the histogram was also centred around a 1:1 orf:protein length. No substantial indel problem was detected in the assembly. The same result was obtained for the plasmid carrying the *bla*$_{\text{TEM}}$.

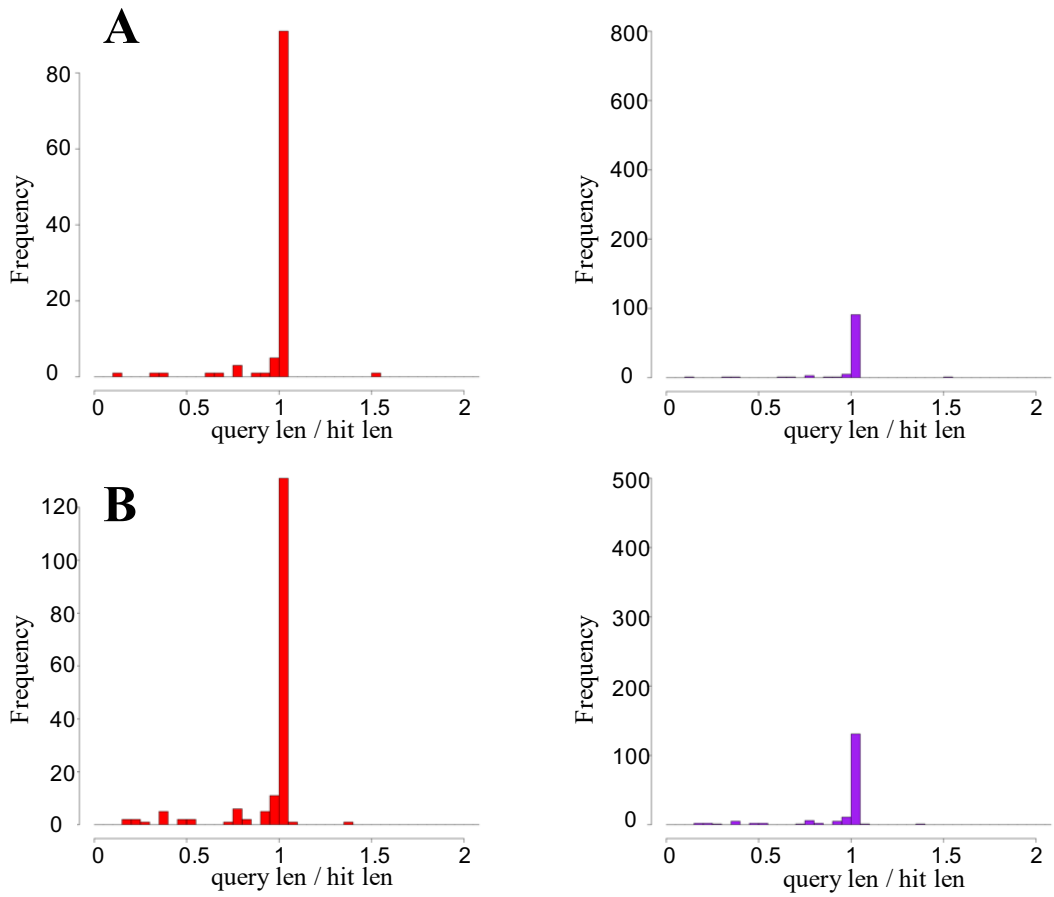


Figure 2.5 Indel plots depicting called ORF length: database hit length. Plots are centred at 1:1 for the plasmid carrying the *bla*$_{\text{CTX-M-15}}$ (**A**) and the *bla*$_{\text{TEM}}$ (**B**).

2.3.3.3 Plasmid map

pSRJ48c plasmid is a 92, 349 bp and was classified as an IncB/K/O/Z plasmid. IncK, IncB/O and IncZ belongs to the I-plasmid complex (IncI). It contained a large transfer region (*tra* genes) important for the transfer and conjugation of the plasmid, a TA system: the RelE/ParE. Two genes *psiA*/*psiB,* involved in the SOS response were also identified. The type 2 secretion system (T2SS) was also genetically characterised in this system. The only ARG present on the plasmid was *bla*$_{\text{CTX-M-15}}$ which was in close

proximity to a transposase and to an IS element identified as IS5 (Figure 2.6). A complete list of genes identified can be found in Appendix 1.

The IncFIB/IncFII plasmid, pSRJ48t is an 150,763 bp circular plasmid which included a transfer operon (*tra* genes) and the virulence genes; *cvaA*, *cvaB*, *cvaC*. These specific genes are involved in the production of a toxin called colicin V. A hemolysin factor *hlyF*, *estA, estB, estC*, *ompT* an adhesine factor, *ironB/C/D/E/N* a siderophore encoding genes were also identified within the plasmid backbone (Figure 2.7). In contrast with pSRJ48c, a vastly different ARGs profile was identified on this plasmid which include the *bla*$_{\text{TEM}}$, *sul2*, *aph(3")-lb*, *dfra17* and *aph(6)-id*. Numerous MGEs were also identified within the sequence assembly which include; IS629, IS66, IS2, IS66, ISEc32. The plasmid also carried the mobilizable elements *Int2,* found next to the *dfra17* genes, and a Tn21 transposase. All the genes identified on this plasmid can be found in Appendix 2.

bla$_{OXA}$ was not identified on any of the two plasmids, analysis of the WGS showed that this β-lactam resistance gene was chromosomally encoded and clearly integrated into the host chromosome. All the ARGs and efflux pumps identified on the chromosome can be found in Appendix 3.

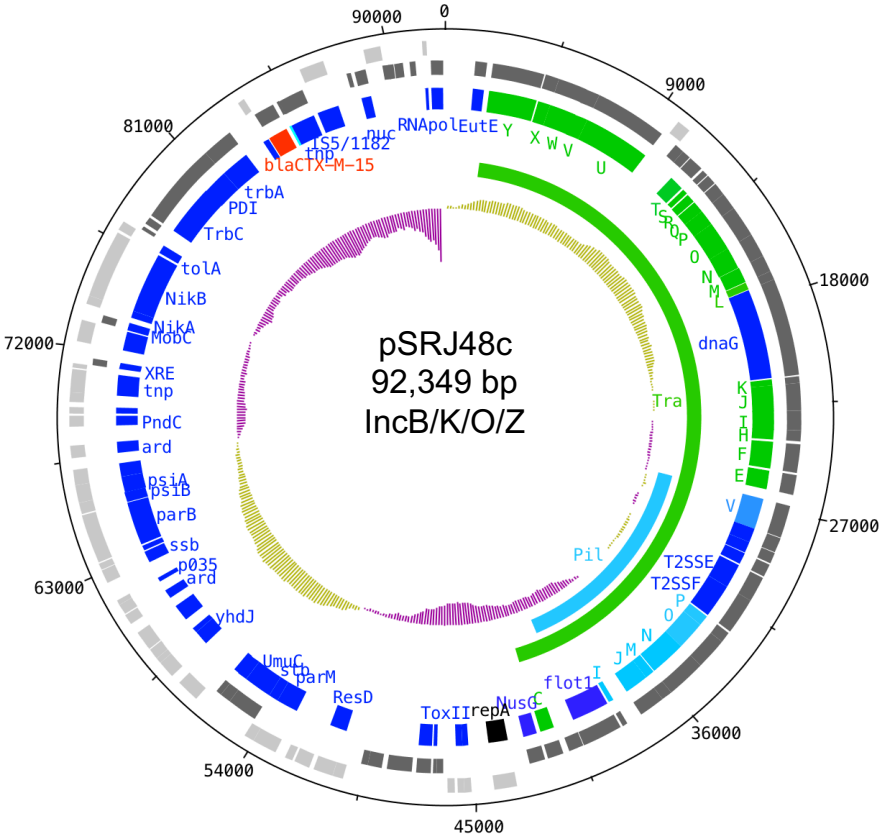


Figure 2.6 Functional modules identified on the pSRJ48c carrying the *bla*$_{\text{CTX-M-15}}$.

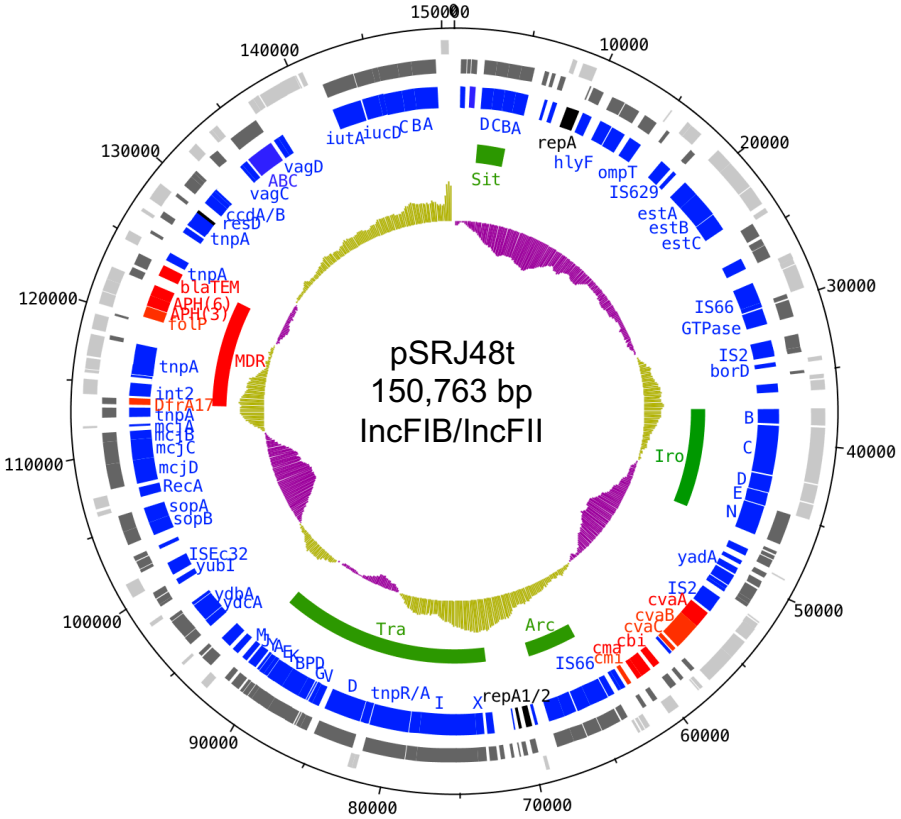


Figure 2.7 Functional modules identified on the pSRJ48t carrying the *bla*$_{\text{TEM}}$.

2.4 Discussion

The use of ONT long-read sequencing has facilitated sequence acquisition, assembly and annotation of the *bla* genes which was previously unachievable using the existing short-reads sequence data set (Hill, 2016). The resistance genotype, conjugative behaviour, Inc group and structure of each individual plasmid with identification of the genetic location of ARGs were determined using a precise workflow including hybrid assembly using the existing short-read Illumina data. Evaluation of the indels indicated approximately 5 % error in ORF structure which can be explained by the presence of pseudogenes in *E. coli.* Studies have reported that pseudogenes occupied 1-5 % of prokaryotic genomes (Liu *et al.*, 2004). The two plasmids identified were carried by *E. coli* ST131, a strain type known to be a highly virulent globally distributed human pathogen which has been identified as being responsible for the dissemination of the $bla_{\text{CTX-M-15}}$ gene worldwide. *E. coli* ST131 is often associated with UTIs and bloodstream infection in both humans and domestic animals, as well as neonatal meningitis and nosocomial pneumonia (Woodford *et al.*, 2009; Can *et al.*, 2015; Hertz *et al.*, 2016). Phylogenomic studies have shown that ST131 are clustered into three clades referred to as A, B and C, the last of which is most associated with the dissemination of $bla_{\text{CTX-M-15}}$ (Petty *et al.*, 2014; Stoesser *et al.*, 2016). Analysis performed by Nabil-Fareed Alikhan using the EnteroBase database and the known genome of MDR clones within the ST131 identified *E. coli* 48 as a member of clade A (Figure 2.8) (Petty *et al.*, 2014; Zhou *et al.*, 2019).

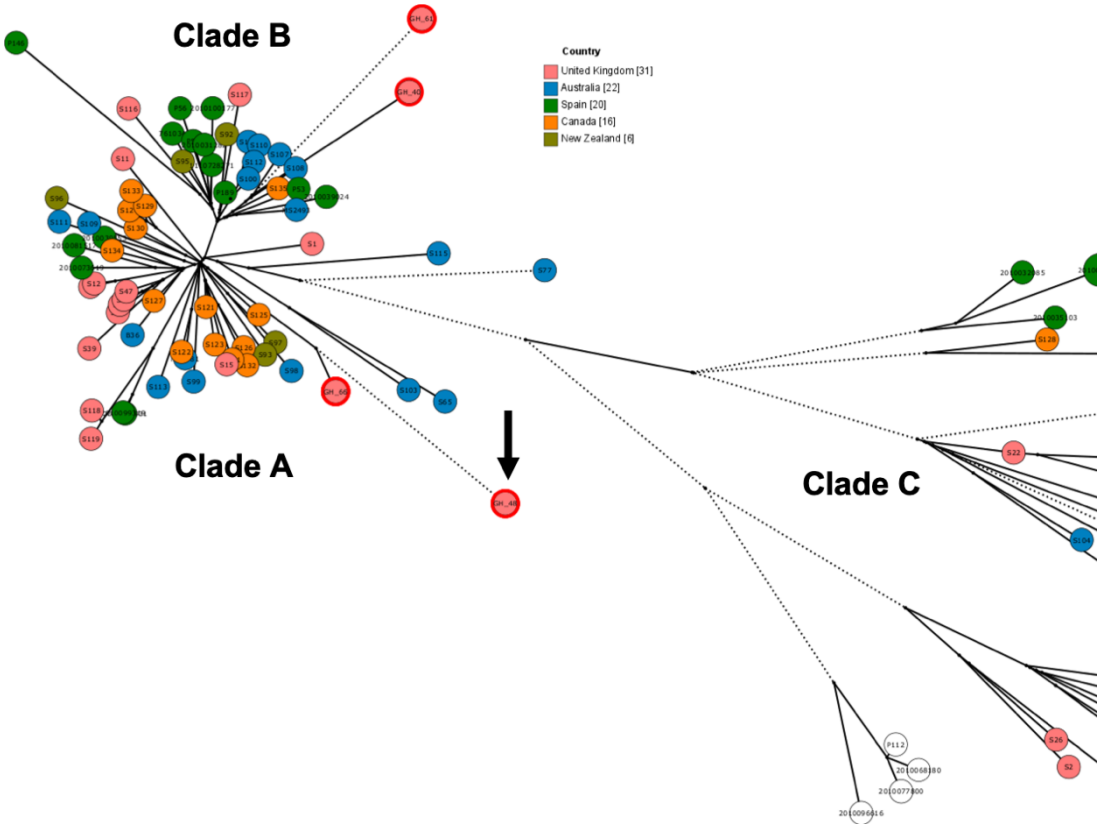


Figure 2.8 Phylogenic three using GrapeTree from EnteroBase identified strain 48 (pointed by the arrow) in the Clade A (Zhou *et al.*, 2019).

The pSRJ48c carried only one ARG, the *bla*$_{\text{CTX-M-15}}$, which contrasted with a previous study that reported *bla*$_{\text{CTX-M-15}}$ genes were often associated with other resistant determinant such as *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$ and *aac(6)-Ib-cr* (Carattoli, 2009). Previous studies have shown that the *bla*$_{\text{CTX}}$ is often associated with the IncF replicon (Gonullu *et al.*, 2008; Carattoli, 2009; Marcade *et al.*, 2009; Lee *et al.*, 2011). In addition, IncI plasmids were also identified in *E. coli* and Salmonella strains which contain *bla*$_{\text{CTX-M-15}}$ (Johnson *et al.*, 2011). In this study the *bla*$_{\text{CTX-M-15}}$ was found in close proximity to an IS element identified as ISEcp1, which is the most common element associated with *bla*$_{\text{CTX-M-15}}$ (Partridge *et al.*, 2011; Zhao and Hu, 2013; Zong *et al.*, 2015). Next to the ISEcp1, the IS5 was identified, which has 99 % amino-acid similarity to another insertion element called ISKpn26. Those two IS elements play a role in the mobility of the *bla*$_{\text{CTX-M-15}}$ as they are able to move themselves and the associated ARG to a new location in the same or different DNA molecule within a cell (Canton and Coque, 2006; Dhanji *et al.*, 2011b; Amos *et al.*, 2014). It has been reported that ISEcp1 element was responsible for capturing different ARGs from diverse bacteria and was

shown to provide a promoter for expression of the *bla*$_{\text{CTX-M-15}}$ (Zong *et al.*, 2015). Further work is needed to confirm the function of the promoter on enzyme expression. Conjugation contributes to the dissemination of the ARG containing plasmids and is likely responsible for the explosion of the multi-drug resistance among clinically significant pathogens (De La Cruz and Davies, 2000). A previous study by Amos *et al.* (2014) demonstrated the ability of *E. coli* isolated from the river sediment to transfer the *bla*$_{\text{CTX-M}}$ on a IncF plasmid to a lab strain (Amos *et al.*, 2014). The *tra* genes are often conserved due to their essential role in the conjugation and the transfer of plasmids, and thus genes, within Gram-negative and Gram-positive bacteria. A functional set of *tra* genes for *E. coli* was found in the plasmid carrying the *bla*$_{\text{CTX-M-15}}$. The plasmid pSRJ48c showed the presence of different *tra* genes involved in the T4SS that allows pilus formation and the creation of a pore where the plasmid DNA will be transported (Cascales and Christie, 2003). In contrast, *traS* and *traT* are two other important genes carried on this plasmid and have a function called surface exclusion, a phenomenon that creates a barrier against conjugative transfer into bacterial cells that have a closely related plasmid (Achtman *et al.*, 1977; Garcillan-Barcia and de la Cruz, 2008). The *traS* reduces the receptiveness to the pilus and the TraT stays in the inner membrane to prevent DNA entry. TraT plays a role in virulence by decreasing *E. coli* sensitivity to phagocytosis by macrophages (Garcillan-Barcia and de la Cruz, 2008). Functional modules were identified on the pSRJ48c that contribute to the stability to kill cells that fail to inherit a copy of the plasmid if partitioning fails. As an example, many plasmids encode TA; the antitoxin component inhibits the expression or the activity of the toxin, where daughter cells that do not acquire a plasmid will have toxin in their cytoplasm but no mechanism to produce the antitoxin resulting in cell death (Hayes, 2003; Goeders and Van Melderen, 2014). The RelE/ParE was found on the pSRJ48c, ParE is involved in the partition system, and will act by inhibiting the DNA gyrase and RelE is involved in the bacterial stress response, then cells will be able to enter a dormant state in order to evade killing through stress (Harms *et al.*, 2018). The TA RelE/ParE system identified in pSRJ48c will contribute to the stable maintenance of the plasmid in absence of cephalosporin selective pressure but may be influenced by plasmid's fitness related genes on the host chromosome and also by changes on the plasmid genome. In the natural community, rapid compensatory mutations in the chromosome and/or in the plasmid overcome the cost involved by the plasmid and enhance the fitness and allow survival of the plasmid

(San Millan *et al.*, 2015; Harrison *et al.*, 2016; Loftie-Eaton *et al.*, 2017; Hall *et al.*, 2020). The plasmid may then provide a selective advantage and increased fitness through the carriage of ARGs.

E. coli express specific genes on exposure to UV or chemical reagents that damage DNA, these are generally repressed and referred to as the SOS response. Plasmid annotation showed the presence of SOS response genes *impA* and *impC* on the pSRJ48c. In the environment where UV radiation or other chemical agents can be found, strains containing the *imp* will have a selective advantage allowing the maintenance of the virulence plasmid pSRJ48c in the external environment. This will also be true for exposure to biocides in the hospital environments and detected in WWTP effluent (Gillings *et al.*, 2008; Pal *et al.*, 2015; Singer *et al.*, 2016; Bengtsson-Palme *et al.*, 2018). Badgasarian *et al*. (1986) showed that *psi* genes can affect the generation of this SOS signal and permit the transfer of the single-stranded plasmid, followed by second strand synthesis in the recipient (Bagdasarian *et al.*, 1986; Wolkow *et al.*, 1996). During the conjugation, a single strand of the plasmid is injected into the recipient cell and this is able to trigger the SOS response independently of external UV or chemical stimuli. The *psiA* and *psiB* were present on pSRJ48c, expression of which results in repression of the SOS response, thus permitting second strand synthesis of the transferred DNA (Althorpe *et al.*, 1999). Genes encoding the T2SS were also found on the plasmid, allowing secretion of folded proteins from the periplasm into the extracellular milieu and this system is important for pathogenic and non-pathogenic bacteria (Korotkov *et al.*, 2012) (See Chapter 3).

pSRJ48t is larger than pSRJ48c at 150,763 bp. This plasmid was classified as an IncFIB/IncFII plasmid which have been commonly detected in both clinical and environmental *Enterobacteriaceae* strains and are a substantial contributor to transmission of ESBLs genes around the world (Carattoli, 2008; Carattoli, 2013). IncF plasmids are low-copy plasmids which often carry an array of ARGs and have consequently been detected in many clinical isolates but have also been isolated from animals (Carattoli, 2009). The MDR region identified on pSRJ48t provides resistance to antibiotics that have been used in both clinical and veterinary settings; the bla_{TEM} provides resistance to β-lactam, *sul2* to sulfonomide, *dfrA17* to trimethoprim and both *aph(6)-id; aph(3")-lb* to aminoglycoside streptomycin. The co-localisation of these

ARGs on the same plasmid may allow the rapid spread across bacteria (Huddleston, 2014; von Wintersdorff *et al.*, 2016). pSRJ48t contained multiple IS elements suggesting a highly mobilizable resistome and these will often enable continuous expression of ARGs due to a location in close proximity to a promoter (Depardieu *et al.*, 2007). For example, the bla_{TEM} was found next to a transposase, and a class 2 integron (*IntI2*) was identified in the MDR cassette. The presence of transposase elements may enhances its role in the integration and dissemination of ARGs cassettes (Ramirez *et al.*, 2010). The co-carriage of *sul2* and *dfrA17* on an integron on pSRJ48t agrees with previous studies on clinical isolates as both trimethoprim and sulfamethoxazole were used in combination to treat UTIs (Blahna *et al.*, 2006). These are both target enzymes in the vitamin B pathway and represent mutations which have a lower binding affinity for the substrate compared to the wildtype on the chromosome. Nevertheless, according to the literature, the *IntI2* cannot acquire new gene cassette due to a non-functional *IntI* gene but the other transferable elements such as the transposons present on the plasmid can play a role in HGT (Bennett, 1999). Two replicons where found in the plasmid, RepA and RepA1/2, it has been reported that in multireplicon plasmids, one replicon is strongly conserved because of the selective pressure imposed where the other can diverge (Sykora, 1992). Similar to the previous plasmid, pSRJ48t also contained a *tra* operon allowing it to be conjugative. TraD, TraP are conjugal transfer proteins, TraL, TraK, TraE and TraB are involved in the pilus assembly and TraI is known to be a catalase that nicks the DNA at the *oriT*. Different VFs are related to the pathogenicity of ExPEC and have a wide range of activities including bacterial colonization by release of adhesins, disabling host defencing by secretion of toxins and iron acquisition. In a mammalian host, iron is in short supply so genes encoding for high-affinity iron-transport allowed the bacteria to colonize the host and caused infection (Miethke and Marahiel, 2007). SitABCD is an ABC type transporter and transports Fe to the cell interior (Sabri *et al.*, 2008). *E. coli* are equipped with siderophores that increase their virulence termed enterobactin, salmochelin, yersiniabactin, aerobactin. *ironBCDEN* gene cluster found on the plasmid is known to encode salmochelin, and *iutA/iucABCD* an aerobactin contributing to the virulence of the host. pSRJ48t also contained genes involved in the production of VFs such as *cvaA*, *cvaB*, *cvaC*, facilitation colonization involved in the production of a toxin called colicin V, a small protein that will disrupt the formation of cell membrane potential that it is required for energy production. The ColV operon

consists of genes for ColV synthesis (*cvaC*), and two genes for the export (*cvaA*, *cvaB*) (Gilson *et al.*, 1987; Waters and Crosa, 1991). *hylF* gene encoding for a hemolysin was identified, this gene has been shown to be expressed during extraintestinal infection and promotes VFs involved in the production of OMVs in ExPEC and in particular APEC (Murase *et al.*, 2016). ompT acts as an outer membrane endoprotease and can cleave protein residues involved in membrane protein. A study showed that the prevalence of *iroN*, *iutA*, *ompT* and *hlyF* genes were more frequently detected in clinical ExPEC isolates compared with commensal isolates, which highlights the selective advantage inferred by environmental plasmids to ST131 and ExPEC strain types (Cyoia *et al.*, 2015). Carattoli (2008) reported that the acquisition of ARG on virulence plasmids may be a novel tool in bacterial evolution to explore and colonise novels hosts as the IncF plasmids are limited by host range to *Enterobacteriaceae* (Carattoli, 2008).To summarise, pSRJ48t is a mosaic of virulence genes, transfer genes, ARGs and MGEs.

The third β-lactamase gene $bla_{\text{OXA-1}}$ was found to be encoded on the chromosome. This contradicts a majority of previous studies which reported that this penicillinase was identified as being commonly plasmid-borne and carried by a large number of Gram-negative bacteria. It was also reported that $bla_{\text{OXA-1}}$ is most frequently found in the context of a gene cassette flanked by integrons (Zhou *et al.*, 1994; Fluit and Schmitz, 2004; Poirel *et al.*, 2010). Studies reported that plasmids harbouring the $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-15}}$ have been identified in human *E. coli* isolates (Coque *et al.*, 2008a). It is likely that the transposase flanking this gene was fundamental in the stable insertion of the gene onto the historical chromosome.

The workflow developed in this study using ONT technology has facilitated the characterisation of the two plasmids present in the host *E. coli* strain 48. The acquisition of AMR and VFs on the plasmids played a role in the global spread of this ST131 clone. Amos *et al.* (2014) showed that the carriage of multiple plasmids of different Inc groups will contribute to higher conjugation rates (Amos *et al.*, 2014).

Chapter 3:

Expression and stability of *bla*$_{CTX\text{-}M\text{-}15}$ in degradation of β-lactam antibiotic

3.1 Introduction and aims

In the ESBL group, CTX-M has the largest number of variants with CTX-M-15 and CTX-M-14 being the predominant genotypes, and the CTX-M-14 shares >80% amino acid identify with CTX-M-15 (Coque *et al.*, 2008a; Hawkey and Jones, 2009; Dolejska *et al.*, 2011). The two enzymes sharesThe prevalence of CTX-M ESBLs has increased since 2000, which has created a huge challenge to healthcare services by greatly limiting the options available for successfully treating bacterial infections caused by CTX-M-producing bacteria (Woerther *et al.*, 2013). Previous work in the Wellington Lab reported increased bacterial resistance to antibiotics and revealed a high diversity of mobile resistance genes in *E. coli* collected downstream of a WWTP from the River Sowe (UK) (Amos *et al.*, 2014; Amos *et al.*, 2018). Subsequently, further work provided numerous antibiotic resistant and sensitive *E. coli* isolates, which were processed for WGS (Hill, 2016). From this collection, two *E. coli* strains were selected on the basis of cefotaxime exposure representing sensitive (strain 33) and resistance (strains 48) phenotypes.

Clinical studies have suggested that secretion of hydrolytic enzymes such as β-lactamases irreversibly inactivate antibiotics outside the cell thus protecting both the producer and other bacteria in the immediate vicinity which would otherwise be sensitive to the antibiotic (Brook, 2004). Based on colorimetric nitrocefin assays, strain 48 was shown to secrete a β-lactamase, but the genes responsible for this clinically important phenotype remain unknown.

In *E. coli*, secretion is achieved through two main pathways: the twin-arginine translocation (Tat) or the general secretory (Sec) pathway. In *E. coli,* the Tat pathway translocate a small number of folded proteins across the cytoplasmic membrane, the majority of proteins are translocated using the Sec pathway (Robinson *et al.*, 2011).

There are six specialized export system in Gram-negative bacteria classified as type 1 secretion system (T1SS), type 2 secretion system (T2SS), type 3 secretion system (T3SS), type 4 secretion system (T4SS), type 5 secretion system (T5SS) and type 6 secretion system (T6SS) (Figure 3.1). These secretion systems ensure the transport of different molecules, in particular, the T3SS is involved in the export of flagellum proteins and toxins, while T4SS injects proteins and nucleic acids acting as VFs or a way of genetic exchange into other cells and T6SS injects pathogenic molecules into other bacterial or eukaryotic targets. Thus, these three secretion systems seem to not be involved in the secretion of antimicrobial resistance enzymes.

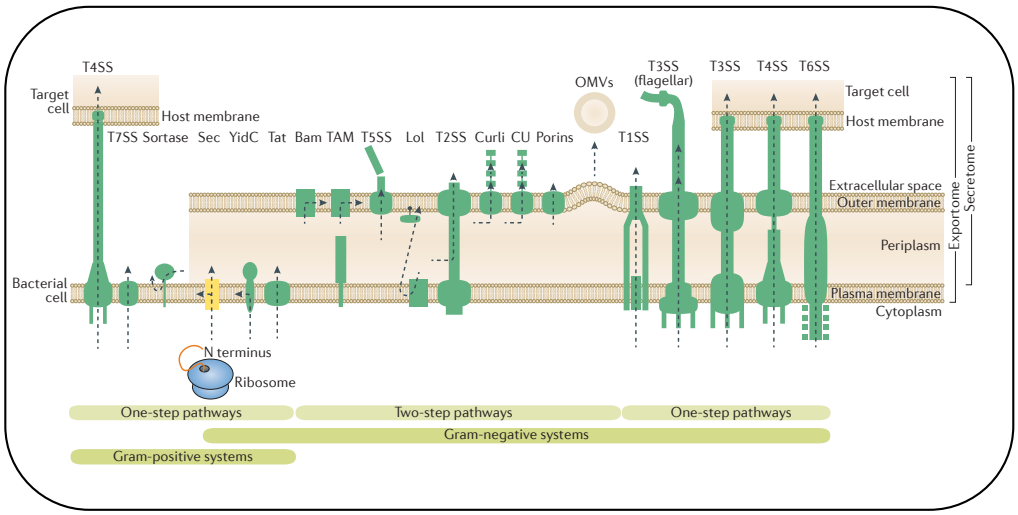


Figure 3.1 The different secretion systems in bacteria. Figure is adapted from (Tsirigotaki *et al.*, 2017).

The architecture of the T1SS is closely related to the resistance nodulation division (RND), an efflux pump associated with resistance to drugs (Piddock, 2006) (Figure 3.2). Whereas, the T2SS ensures the transport of hydrolysing enzymes and toxins. This system includes components called general secretion pathway (Gsp) (Figure 3.2).

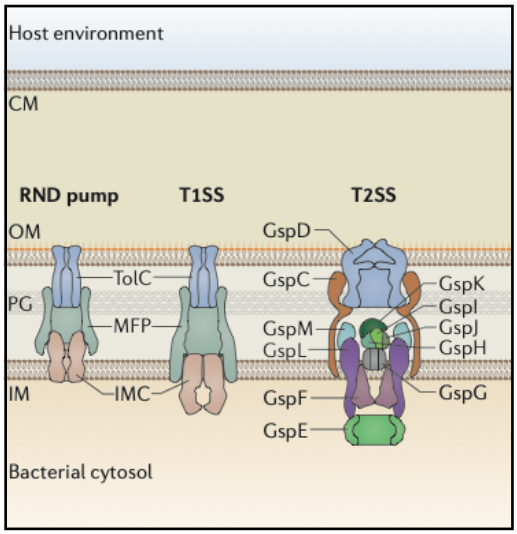


Figure 3.2 Structural organisation of the RND pumps, T1SS and T2SS in Gram-negative bacteria IM: inner membrane, PG: peptidoglycan, OM: outer-membrane, CM: cytoplasmic membrane of hosts cell. Figure is adapted from (Costa *et al.*, 2015).

Gram-negative bacteria have a double membrane envelope composed of an inner membrane, a peptidoglycan layer and an outer membrane rich in lipopolysaccharides which acts as a barrier for secretion thus retaining proteins in the periplasmic space. In contrast, the cell wall of Gram-positive bacteria consists of a single layer of peptidoglycan facilitating the release of proteins into the extracellular environment. *Streptomyces* species, a filamentous Gram-positive bacterial group produce large numbers of extracellular enzymes using signal peptides (Anne *et al.*, 2017).

An alternative phenomenon for the secretion of ESBLs is the formation of outer-membrane vesicles (OMVs) which are common in Gram-negative bacteria (Mashburn-Warren and Whiteley, 2006). The outer membrane and the periplasm are released in the form of spherical, lipid-bilayed vesicles *via* a yet-undefined mechanism. These OMVs vary in size from 10 to 200 nm (Lee *et al.*, 2007). This secretory process eliminates the need for bacterial contact, or complex molecular architectures at the cell wall-periplasm interface allowing long distance dissemination of different proteins. Indeed, packaging of β-lactamases into OMVs has been demonstrated in *Pseudomonas aeruginosa* by microscopy and enzymatic studies (Ciofu *et al.*, 2000). No data is available yet for CTX-M-15, but in addition, it has been shown that secretion of CTX-M-1 from OMVs in a *E. coli* obtained by conjugation between a laboratory strain antimicrobial-sensitive *E. coli* RC85 with a multidrug resistant *Salmonella* spp, Sal45 allowed survival of antibiotic susceptible

bacteria in presence of β-lactam antibiotics such as ampicillin, cefotaxime and cefoperazone (Kim *et al.*, 2018). Vesicles were isolated and visualized using transmission electron microscopy, however, the mechanism of β-lactamase production and secretion in *E. coli* remains elusive. The study of NDM-1, a MBL that has rapidly evolved due to the extensive use of carbapenems in clinical medicine with the spread of clones carrying bla_{NDM-1} on MGEs (Poirel *et al.*, 2011). The gene bla_{NDM-1} has a lipido box motif in its sequence which enables proteins to traverse the cell wall as opposed to remaining in the periplasm (King and Strynadka, 2011; Kovacs-Simon *et al.*, 2011). Without this another mechanism must be invoked for movement from the periplasm across the peptidoglycan and beyond the outer cell membrane. Lipidated NDM-1 is allowed to anchor to the outer-membrane of Gram-negative, contributing to the stability of the enzyme and its secretion through OMVs (Gonzalez *et al.*, 2016).

The aim of this study was to determine if strain 48 could provide a protective effect to susceptible bacteria when challenged with cefotaxime through the production and secretion of ESBLs. The hypothesis being tested was that strain 48 secreted CTX-M-15 through either a molecular secretory mechanism, such as the T1SS or T2SS, or OMVs. The key aspect of β-lactamase activity with strain 48 was to prove the presence of enzyme in the exoproteome, which is defined as the proteins that can be found in the extracellular milieu. Without this another mechanism must be invoked for movement from the periplasm across the peptidoglycan and beyond the outer cell membrane. A range of methods are reported here to uncover the mechanism of secretion in strain 48.

3.2 Material and methods

3.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 3.1. Environmental *E. coli* strains were isolated from the River Sowe, Coventry, UK; namely *E. coli* O8:H7 (strain 33) and *E. coli* ST131 O25:H4 (strain 48). Resistance and phenotype profiles for the two environmental strains are listed in Table 3.2 and Table 3.3 (Hill, 2016).

Table 3.1 Bacterial strains and plasmids used in this study.

Plasmid	Description	Reference/Source
pGEM-T	Commercial cloning vector, containing Amp^R and T7 promoter	Promega
pGEM-CTX-M-15	Derivative of pGEM-T, bearing the $bla_{CTX\text{-}M\text{-}15}$ resistance gene driven by a T7 promoter	This study
pGEM-TEM	Derivative of pGEM-T, bearing the bla_{TEM} resistance gene driven by a T7 promoter	This study
pGEM-OXA	Derivative of pGEM-T, bearing the bla_{OXA} resistance gene driven by a T7 promoter	This study

Strain	Genotype and comments	References/Source
E. coli JM109	*endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac- proAB), [F´ traD36, proAB, laqIqZΔM15]*	Promega
E. coli JM109 pGEM	*E. coli* JM109 bearing the pGEM vector	This study
E. coli JM109 CTX-M-15	*E. coli* JM109 bearing the pGEM-CTX-15 vector	This study
E. coli JM109 TEM	*E. coli* JM109 bearing the pGEM-TEM vector	This study
E. coli JM109 OXA	*E. coli* JM109 bearing the pGEM-OXA vector	This study
E. coli BW 25113	K-12 derivative *rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ*9rhaBAD)568 rph-1* [wild type]	(Datsenko and Wanner, 2000)
E. coli JW5503 Δ*tolC*	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-* , *ΔtolC732::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514* [wild type]	(Baba *et al.*, 2006)
E. coli BW 25113 JW5707 ΔgspD	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-* , *ΔgspD735::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514* [wild type]	(Baba *et al.*, 2006)
E. coli BW+	Derivative of 25113carrying pGEM-T -$bla_{CTX\text{-}M\text{-}15}$	This study
E. coli Δ*tolC*+	Derivative of 25113carrying pGEM-T-$bla_{CTX\text{-}M\text{-}15}$	This study
E. coli JW5707 ΔgspD+	Derivative of 25113carrying pGEM-T-$bla_{CTX\text{-}M\text{-}15}$	This study

Table 3.2 Resistance profile for the isolates from the river Sowe genotypes.

Isolate	ST	β-lactams	Vancomycin	Sulfonomide
33	3576	/	/	/
48	131	$bla_{CTX\text{-}M\text{-}15}$ bla_{TEM} bla_{OXA}	*vanG*	*sul1*

Table 3.3 Phenotypic resistance profile of strains 33 and 48.

Isolate	Ampicillin (25 µg	Cefotaxime (5 µg)	Imipenem (10 µg)	Chloramphenicol (30 µg)	Erythromycin (8 µg)
33	No	No	No	No	No
48	Yes	Yes	No	No	Yes

Cells were routinely grown in LB liquid broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or on LB solid (addition of 15 g/L agar) medium, supplemented with 8 µg/ml of cefotaxime, 100 µg/ml of ampicillin, 5 µg/ml of kanamycin when required. Additionally, culture medium was supplemented with isopropyl β-D-thiogalactosidase (IPTG) (0.1 M) and X-galactosidase (20 mg/ml). Cells were incubated at 37ºC with either shaking (200 rpm) or static conditions. All *E. coli* strains were stored in 8% (v/v) glycerol at -80ºC.

3.2.2 Cloning and expression of $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM,}$ and bla_{OXA} in pGEM-T Easy vector

The primers used to amplify the β-lactamase genes $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM} and bla_{OXA} from strain 48, are listed in Table 3.4. PCR reactions were done using 12.5 µl Master Mix 2X (Promega), 1.25 µl DMSO, 0.8 µM forward primer, 0.8 µM reverse primer, 2 µl DNA template and dH_2O for a final PCR reaction volume of 25 µl. PCR was performed at an initial denaturation temperature at 95ºC for 5 min, followed by 34 cycles of denaturation at 95ºC for 30 sec, annealing temperature at 55-66ºC, (depending on the primer set) for 30 sec and extension for 1 min 50 sec. A final extension was performed at 72ºC for 5 min. The PCR product was purified by using the QIAquick® PCR Purification kit (Qiagen). The purified PCR was A-tailed, ligated into pGEM-T easy vector (Promega) (Table 3.1) and then transformed into *E. coli* JM109 cells following the manufacturer's instruction (Promega), resulting in the new vectors pGEM-CTX-M-15, pGEM-TEM and pGEM-OXA (Table 3.3). The newly constructed plasmids were transformed into *E. coli* JM109, resulting in the strains *E. coli* JM109 CTX-M-15, TEM and OXA (Table 3.3). A control *E. coli* JM109 strain possessing the empty pGEM-T vector was also generated.

Table 3.4 Details of primers pairs used in this study for PCR screening and cloning.

Target	ID	Sequence	Ta (°C)	Size (bp)	Reference
bla$_{CTX-M-15}$	CTX-M_F	5'ATGGT AAAAA TCACT CGCCA GTTC3'	63	876	This study
	CTX-M_R	5'TTACA AACCG TCGGT GACGA T3'			
bla$_{TEM}$	TEM_F	5'ATGCG CCTGG TAAGC AGAGT3'	55	1124	This study
	TEM_R	5'TTACC AATGC TTAAT CAGTG3'			
bla$_{OXA}$	OXA_F	5'CAGTT ACTGG CGAAT GCATC3'	66	1287	This study
	OXA_R	5'CGTCC CGACT TGATT GAAG3'			
kan	k2	5'CGGTGCCCTGAATGAACT GC3'	58	471	(Datsenko wanner 2000)
	kt	5'CGGCCACAGTCGATGAAT CC3'			
gspD735	gspD_F	5'CGATCCTGATCGACCCT3'	52	505	This study
	gspD_R	5'TTACCGTGGTTTCGCTCAT TCG3'			
tolcC732	tolC_F	5'ATGCAAATGAAGAAATTG CTCCCCATTCTTATCG3'	58	1482	This study
	tolC_R	5'TCAGTTACGGAAAGGGTT ATGACCGTTAC3'			

3.2.3 Preparation of calcium competent cells and electro transformation

E. coli BW 25113 (*E. coli* BW), *E. coli* JW5503 Δ*tolC* (*E. coli* ΔtolC) and *E. coli* JW5707 ΔgspD (E. coli ΔgspD) were incubated o/n in three different flasks containing 5 ml of LB at 37 °C at 200 rpm. 50 µl of the o/n culture were inoculated into 5 ml of LB. *E. coli* BW, *E. coli* ΔtolC and *E. coli* ΔgspD were harvested at an A_{600} nm of 0.4-0.5 from a mid-logarithmic phase of growth. The cells were collected by centrifugation at 8000 rpm for 2 min at room temperature and the pellets were gently resuspended in 200 µl of ice-cold 1M $CaCl_2$. This step was repeated. 100 µl of cell suspensions was used for each transformation. Plasmid DNA, pGEM-CTX-M-15 was added to the cells and incubated on ice for 30 min. The mixture was then transferred to a 0.2 cm electroporation cuvette and pulsed at 2.5 kV with a BioRad gene pulser. 1 ml of SOC outgrowth medium (New England BioLabs) was added to the cuvette and transferred to an Eppendorf tube. The tube was incubated for 1 h at 37°C at 200 rpm. Transformants were then selected on LB agar containing 100 µg/ml of ampicillin and 50 µg/ml of kanamycin. Successful transformants *E. coli* BW+, *E. coli* ΔtolC+ and *E.*

coli ΔgspD+ were checked by PCR for the presence of the plasmid (primers in Table 3.4). A control strain was obtained transforming *E. coli* BW, *E. coli* ΔtolC and *E. coli* ΔgspD with the empty pGEM-T vector.

3.2.4 Growth curves

Growth measurement were carried out in 96-well plates with 200 µl culture per well and incubated at 37°C with shaking (200 rpm) in a microplate reader (POLARstar Omega, BMG labtech). As inoculum, o/n starter cultures of each bacterial strain (5 ml) were diluted to an initial concentration of 3 x 10^7 cells/ml to a final volume of 200 µl of LB. Culture media was supplemented with 0, 8, 16, 32 or 64 µg/ml of the third-generation antibiotic cefotaxime (VWR International Ltd). Cell proliferation was determined by measuring the optical density at 600 nm for 8 or 12 h every 15 min. Triplicates were used for each condition tested.

Exponential growth rates were calculated for the growth of *E. coli* BW+, *E. coli* ΔtolC+ and *E. coli* ΔgspD+ and for the protective effect on strain 33 by using the following formula; $P(t) = P_0e^{rt}$ where P(t) was the amount of cell number at time t, P_0 the initial cell number, r the growth rate and t the number of periods (Hall *et al.*, 2014). Two- sample t-Test was performed to compare the significance of the growth rate.

3.2.5 Detection of β-lactamase activity by nitrocefin assay

β-lactamase activity was assessed by colorimetric assay using the chromogenic cephalosporin compound nitrocefin (Thermo Scientific) (O'Callaghan *et al.*, 1972). Nitrocefin in solution is yellow in colour but turn red upon hydrolysis by β-lactamases. Strain 33 and strain 48 were inoculated in LB or M9 minimal media (33.9 g/L Na_2HPO_4, 15g/L KH_2PO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl, 20 % glucose, 1 M $MgSO_4$, 1 M $CaCl_2$) supplemented with 0, 2, 4 or 8 µg/ml cefotaxime. Cells were grown at 37°C with shaking (200 rpm) until log phase and then removed by centrifugation at 4000 rpm for 15 min. The supernatant was then gently filtered through a 0.22 µm filter (Fisher Scientific) to prevent cell lysis. 1 ml of the resulting media was incubated with 15 µg/ml of nitrocefin (stock concentration 500 µg/ml) at room temperature (~22ºC) for 30 min.

3.2.6 Determining expression profiles of *bla*$_{CTX\text{-}M\text{-}15}$ in strain 48

An o/n culture of the strain 48 was diluted into fresh LB media supplemented with 0 or 8 μg/ml cefotaxime. Diluted cultures were grown at 37°C with shaking (200 rpm) to exponential phase before RNA extraction. Total RNA from strain 48 was obtained using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer and the 260/280 and 260/230 ratios were examined to check for protein and solvent contamination. Integrity of RNA samples was confirmed by agarose gel. Contaminant DNA was removed using the TURBO DNA-free kit (Ambion). Total RNA was then reverse transcribed using the SuperScript II Reverse Transcriptase (Invitrogen), following manufacturer's guidelines. A control omitting the reverse transcriptase was carried out for each RNA sample to rule out residual genomic DNA contamination. The obtained cDNA samples were then used as qPCR templates. Expression of *bla*$_{CTX\text{-}M\text{-}15}$ was assessed using the cycle threshold (Ct) values to normalized relative expression to the housekeeping gene *hcaT*, encoding for a 3-phenylpropionic transporter (Zhou *et al.*, 2011). Primers used for *bla*$_{CTX\text{-}M\text{-}15}$ and *hcaT* can be found in Table 3.5. qPCR reactions mixes were prepared as follow: 12.5 μl SYBR® GREEN master mix (Invitrogen), 0.4 μM forward primer, 0.4 μM reverse primer, 0.5 mg/ml BSA, 8.25 μl dH_2O, and 1 μl DNA template. Reactions were carried out in the 7500 Fast Real-Time PCR System (Applied Biosystem), with the following conditions: (1) initial denaturation at 95^{o}C for 10 mins, (2) denaturation at 95^{o}C for 15 sec, (3) annealing at 60^{o}C for 1 min. Step 2 and 3 were repeated for 40 cycles.

Table 3.5 Target amplified using qPCR.

Target	Name	Sequence	Reference
blaCTX-M-15	CTX-M-15OF	5'ATGGTAAAAAATCACTGCCAGTTC3'	This study
	CTX-M-14OER	5'TTACAAACCGTCGGTGACGAT3'	
hcaT	hcaT_F	5'GCTGCTCGGCTTTCTCATCC3'	(Zhou *et al.*, 2011)
	hcaT_R	5'CCAACCACGCTGACCAACC3'	

3.2.7 Testing the protective effects of β-lactamase producing strain on susceptible strain in the presence and absence of cefotaxime

Cefotaxime susceptible strains (strain 33) were first grown o/n at 37ºC at 200 rpm. The resistant strain 48, JM109 pGEM-CTX-M15, -TEM and -OXA, *E. coli* BW+, *E. coli* ΔtolC+ and *E. coli* ΔgspD+ were grown separately and centrifuged at 4000 rpm for 15 min. The supernatant was filtered through a 0.22 μm filter (Fisher Scientific), resulting in what will be referred as conditioned medium (CM) in the rest of this Chapter. Cells of the susceptible strain were inoculated in either fresh LB medium or a mixture of CM: fresh LB (1:3 ratio). Liquid media were supplemented with 0, 8, 16, 32, 64 μg/ml cefotaxime. Cell proliferation was determined by measuring the OD at 600 nm for 12 h every 15 min using a microplate reader (POLARstar Omega, BMG labtech), where cells were incubated at 37ºC with shaking (200 rpm). Triplicates were used for each condition tested.

3.2.8 Preparation of exoproteome, total proteome samples and LC-MS/MS analysis

Exoproteomes and total proteomes of strain 48 were prepared as previously described in Christie-Oleza and Armengaud (Christie-Oleza and Armengaud, 2010), with the following modifications; cells were grown until log phase in M9 minimal medium at 37°C with shaking (200 rpm) and then centrifuged at 4,000 rpm for 15 min at 4°C. Supernatants were then filtered through two consecutive low-proteins-binding FisherBrand™ sterile PVDF filters (0.45 μm filter, followed by 0.22 μm filter) (Fisher Scientific) and acidified at pH 5 using a solution of trifluorocetic acid 10% (v/v). 50 ml of supernatant was incubated o/n on a rotor wheel (40 rpm/min) with 30 μl of Strataclean beads (Agilent). Beads were collected by centrifugation (2,000 rpm for 1 min) and resuspended in lithium dodecyl sulfate (LDS) (Expedeon Ltd) amended with

the reducing agent, dithiothreitol (DTT) (Expedeon Ltd). Sample were heated at 90ºC for 5 min and then sonicated for 5 min. This cycle was repeated twice.
The cell pellets were resuspended in lithium dodecyl sulfate with 1% β-mercaptoethanol. Celle pellets were lysed by using the French Pressure Cell Press.

Purified proteins were separated on a 1D-SDS PAGE (Expedeon Ltd) by protein migration performed at 180 V for 5min. Following protein separation, the obtained gel was stained using SimplyBlue™ SafeStain (ThermoFisher). The stained protein gels were washed three times with dH_2O_2 and then left to destain o/n. Each gel band was then excised and transferred into a sterile microcentrifuge tube. In-gel reduction and alkylation of the proteins were performed using dithiothreitol and iodoacetamide, respectively. Proteins were digested o/n with 40 µl of trypsin solution (2.5 ng/µl) and peptides were recovered using a formic acid/acetonitrile extraction buffer prior to analysis using a nano LC-ESI-MS/MS Ultimate 3000 LC system (Dionex-LC Packings) associated to an Orbitrap Fusion mass spectrometer (Thermo Scientific).
A custom database was made using the genome of strain 48 by using Prokka v1.14.5 for annotation (Seemann, 2014). MASCOT was used to assign peptide to protein by using the custom database, identified proteins were further analysed using Scaffold. The normalized spectral abundance factor was calculated for each protein to compare the abundance for all proteins. Two-sample t-Test was used to determine if presence of antibiotic significantly impacted the proteins abundance.

3.2.9 *In silico* prediction of protein localisation and secretion pathways

The SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP-5.0/) was used to predict the presence and the location of cleavage sites in the three β-lactamase proteins CTX-M-15, TEM, and OXA using the Fasta sequences generated in house (Appendix 4) (Almagro Armenteros *et al.*, 2019).The D-score output is used for discrimination of signal peptide versus non signal peptide with a threshold of 0.570 (Klee and Ellis, 2005). The protein can either have a Sec signal peptide (Sec/SPI), a Lipoprotein signal peptide (Sec/SPII), a Tat signal peptide (Tat/SPI) or no signal peptide.

SecretomeP server (http://www.cbs.dtu.dk/services/SecretomeP/) predicts non-classical secretion but gives a high score to protein entering the classical secretory

pathway (Bendtsen *et al.*, 2005). For each input sequence a score is generated, for which a value above 0.5 indicated possible secretion (Bendtsen *et al.*, 2004)

The TXSScan webtool (https://galaxy.pasteur.fr/) (Afgan *et al.*, 2018) was used for prediction of the presence of secretion systems in strain 48 using the genome of *E. coli* 48 (S, Rangama, Unpublished; Chapter 2).

3.3 Results

3.3.1 The nitrocefin assay on supernatant of *E. coli* 48

An enzymatic assayed based on nitrocefin cleavage by β-lactamase, which causes release of a red colour following hydrolysis of the yellow nitrocefin β-lactam ring, was used to test for the presence of these enzymes in the supernatant of the strain 48 which carries the genes for the ESBLs, bla_{TEM}, bla_{OXA} and $bla_{CTX\text{-}M\text{-}15}$ and strain 33 that does not.

As expected, results of the assay indicated that strain 33 showed no β-lactamase activity in the culture supernatant. The strain 48, however, appeared to secrete active β-lactamases into the medium (Figure 3.3). The colour change was noticeable within 5 min following nitrocefin addition. Secretion of active β-lactamases by strain 48 appeared to be constitutive, as β-lactam hydrolysis was also observed from the supernatant of cells grown without cefotaxime.

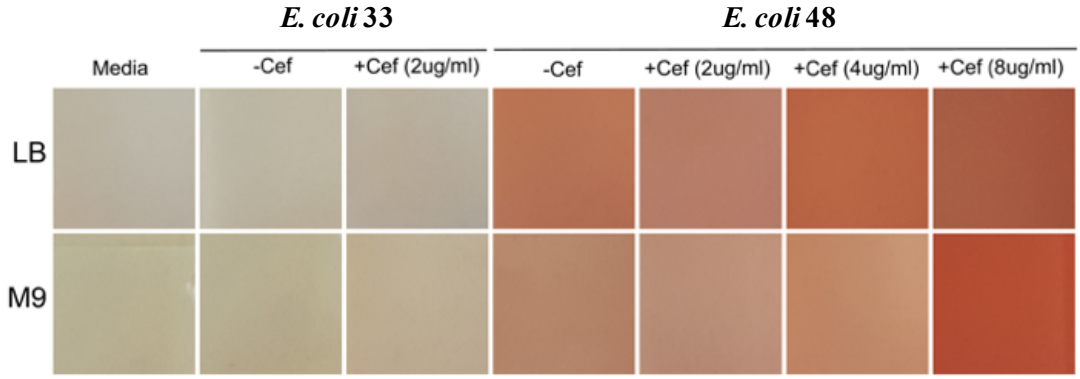


Figure 3.3 Nitrocefin (15 µg/ml) assay with various concentration of cefotaxime (2, 4 and 8 µg/ml) in presence of *E. coli* strain 33 or strain 48.

3.3.2 Influence of various concentrations of cefotaxime on the growth of strain 33 and strain 48

The effect of the presence of cefotaxime was studied by monitoring the growth of strain 33 and 48 over 12 h in absence and presence of cefotaxime (8 µg/ml, 16 µg/ml, 32 µg/ml and 64 µg/ml). In presence of all concentration tested, the growth of strain 33 was inhibited whereas strain 48 was able to proliferate (Figure 3.4). Strain 48 secreted ESBLs in the surrounding environment (Figure 3.3) allowing its growth in presence of cefotaxime (Figure 3.4).

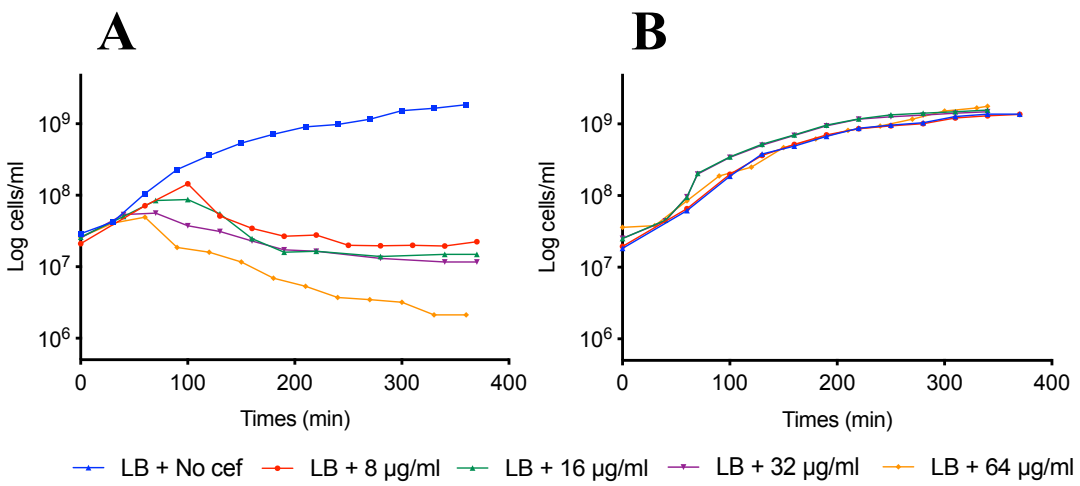


Figure 3.4 Growth of *E. coli* cultivated in LB medium in varying cefotaxime concentrations (8 µg/ml, 16 µg/ml, 32 µg/ml and 64 µg/ml). (**A**) Strain 33. (**B**) Strain 48.

3.3.3 Investigating mRNA expression level of *bla*$_{CTX\text{-}M\text{-}15}$ in strain 48

The expression of the *bla*$_{CTX\text{-}M\text{-}15}$ was investigated by RT-qPCR. Following the addition of cefotaxime (8 µg/ml), the expression of *bla*$_{CTX\text{-}M\text{-}15}$ gene does not change significantly, indicating a constitutive expression of *bla*$_{CTX\text{-}M\text{-}15}$ (Figure 3.5).

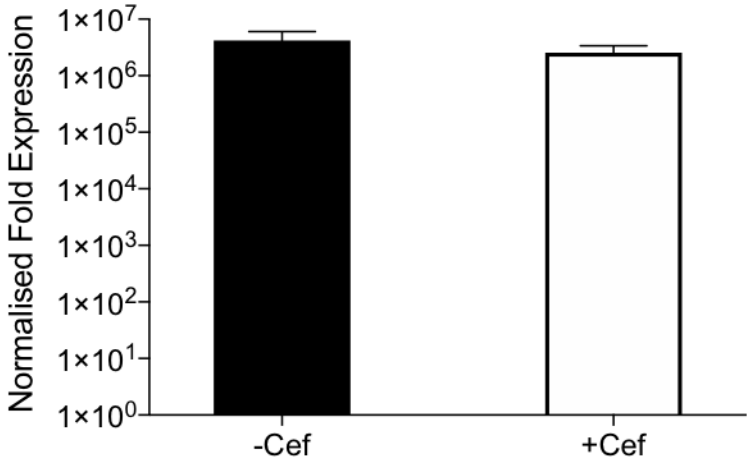


Figure 3.5 Expression of *bla*$_{CTX\text{-}M\text{-}15}$ in strain 48 with and without cefotaxime. No significant difference in transcription following the addition of cefotaxime (8 μg/ml) (p-value=0.3).

3.3.4 Exoproteome of strain 48

As the supernatant of strain 48 tested positive for β-lactamase activity which resulted in resistance to cefotaxime, exoproteomic was employed to identify which of the three ESBLs was responsible for the majority of activity. Comparative proteomics of both the cellular and extracellular protein fractions of cells grown in the presence (8 μg/ml) or absence of cefotaxime was performed.

Both the TEM and the CTX-M-15 β-lactamases were identified in the cellular fraction and also in the exoproteome. Levels of these proteins in the cytoplasmic fraction were however 10 times lower than the ones obtained from the exoproteome, suggesting their active secretion into the extracellular environment (Figure 3.6). Notably, CTX-M-15 was the third most abundant protein detected in the exoproteome of strain 48, with a relative abundance of 2.48 % of the total exoproteome dataset in the absence of cefotaxime and 1.70 % with 8 μg/ml of cefotaxime (Figure 3.6). We also detected the TEM β-lactamase in the exoproteome of strain 48, but with a relative abundance of about 0.38 % in the absence of cefotaxime and about 0.60 % with 8 μg/ml of cefotaxime.

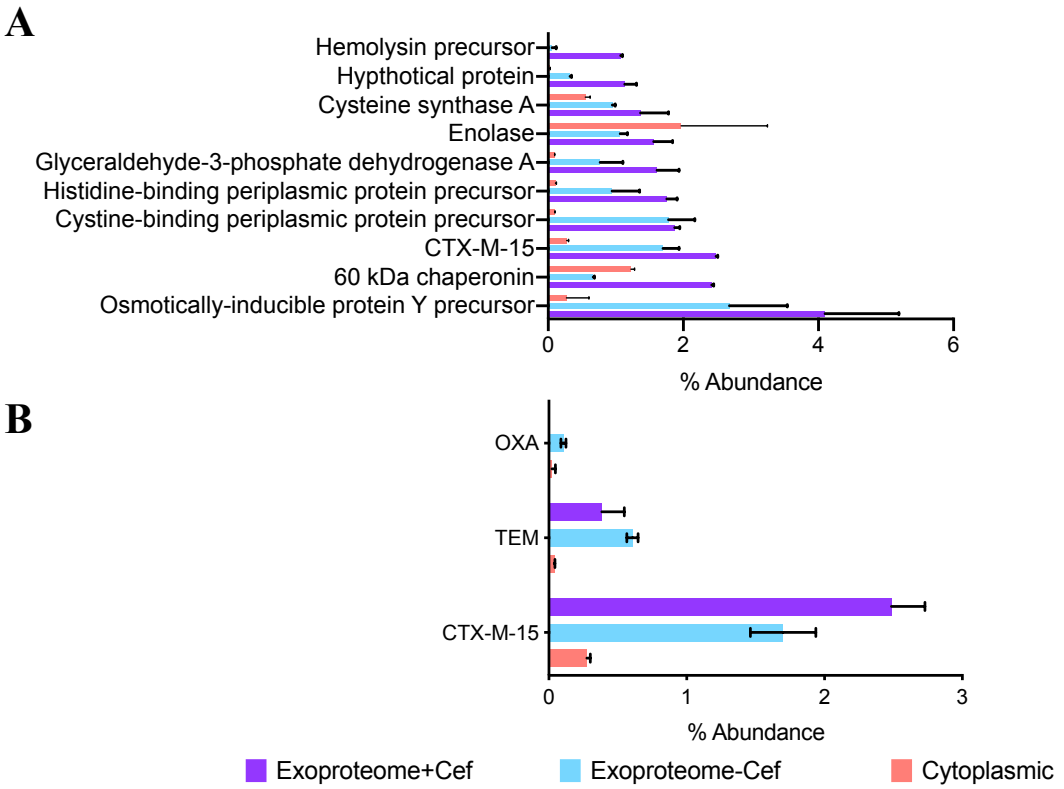


Figure 3.6 Exoproteomes isolated from strain 48. **(A)** Top-10 of the most abundant proteins found in the exoproteome of strain 48 in presence of cefotaxime. Protein abundance was evaluated by MS/MS spectral counts which correlate linearly with the protein abundance. Error bars indicate mean of three replicates. (**B**) Abundance of the three ESBLs proteins. The abundance of CTX-M-15 was significantly higher in presence of cefotaxime (pvalue =0.05).

3.3.5. Identification of the ESBL required for cefotaxime resistance

3.3.5.1 Cloning of the *bla* genes in expression vector in *E. coli* JM109

Proteomic analysis suggested that CTX-M-15 is responsible for conferring cefotaxime resistance in strain 48. To confirm this prediction, $bla_{\text{CTX-M-15}}$ from strain 48 was cloned into the expression vector, pGEM-T easy. Exoproteomics also revealed a small induction of TEM in the exoproteome (relative abundance of about 0.60 % with 8 µg/ml of cefotaxime (Figure 3.6). Therefore, as controls bla_{TEM} or bla_{OXA} were also separately cloned into pGEM-T (Figure 3.7). Plasmids were separately transformed into a susceptible host, the commercial strain *E. coli* JM109, resulting in the strains *E. coli* JM 109 OXA, JM 109 TEM, and JM 109 CTX-M-15.

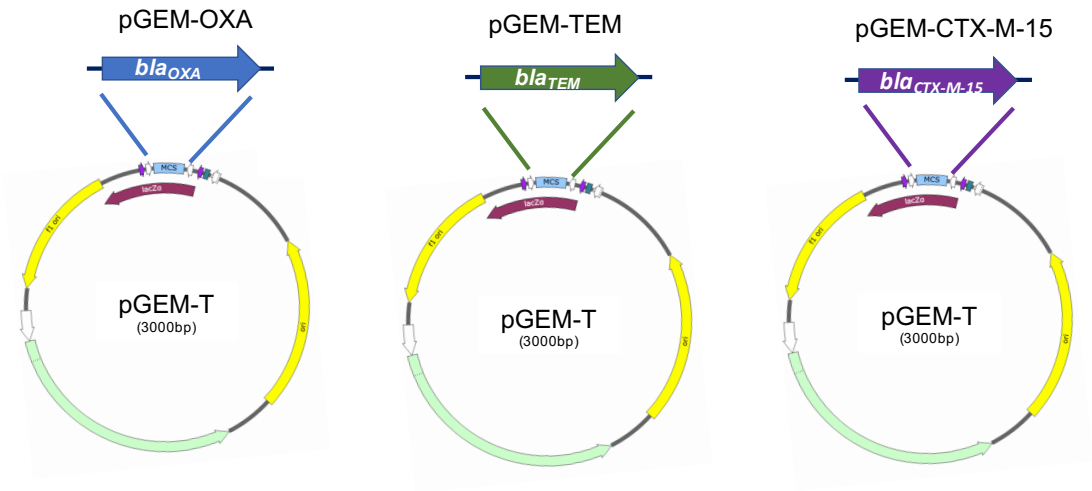


Figure 3.7 PGEM-T vector used for the cloning of the *bla* genes.

E. coli JM 109 OXA, JM 109 TEM, JM 109 CTX-M-15 and JM 109 empty plasmid were grown separately o/n in LB liquid culture in presence of 8 µg/ml of cefotaxime and then plated onto 4 different LB agar plates. Cells were able to grow only for the construct containing the $bla_{\text{CTX-M-15}}$.

The growth of JM 109 empty plasmid and JM 109 CTX-M-15 was monitored over 8 h in absence and presence of cefotaxime (8 µg/ml, 16 µg/ml, 32 µg/ml and 64 µg/ml). In absence of cefotaxime both strains were able to grow but only JM 109 CTX-M-15 proliferated in presence all of concentration of cefotaxime tested (Figure 3.8).

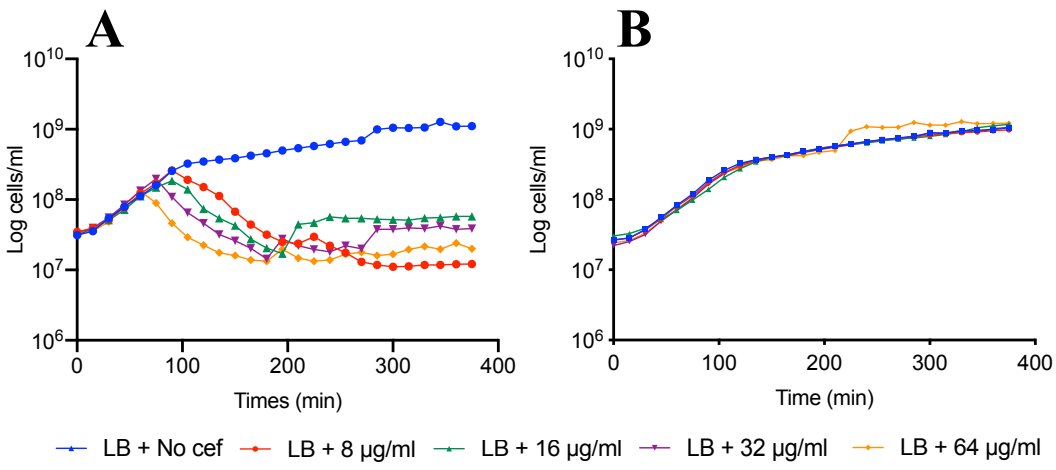


Figure 3.8 Growth of JM 109 cultivated in LB medium in varying cefotaxime concentrations (8 µg/ml, 16 µg/ml, 32 µg/ml and 64 µg/ml). (**A**) JM 109 empty plasmid. (**B**) JM 109 CTX-M-15.

3.3.5.2 Testing the protective effect

3.3.5.2.1 The effect of secreted ESBL strain 48 on a strain 33

Proliferation of strain 33 in fresh LB was monitored over a period of 8 h. As shown previously, strain 33 grew before declined in presence of cefotaxime (8 µg/ml) (Figure 3.9). Strain 33 was incubated in CM obtained from strain 48 in absence and presence of 8 µg/ml of cefotaxime (Figure 3.9). In both conditions, cells were able to proliferate suggesting that strain 48 could provide a protective clearance of the media by secreting β-lactamases in the surrounding environment.

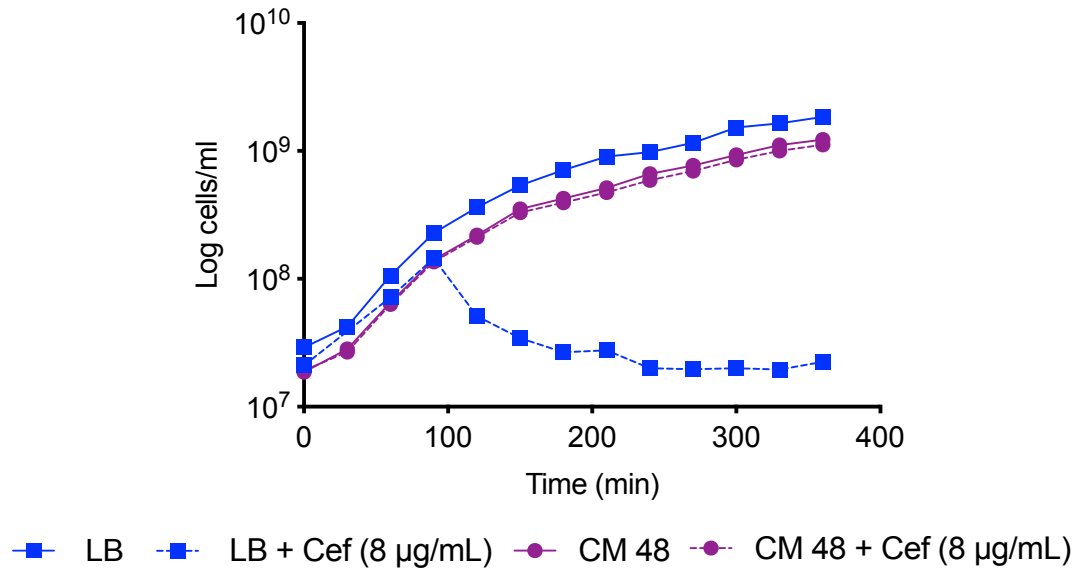


Figure 3.9 Growth of strain 33 cultivated in fresh LB and CM obtained from strain 48, experiment was conducted without or with 8 µg/ml of cefotaxime.

3.3.5.2.2 JM 109 strains as the validation that *bla*$_{\text{CTX-M-15}}$ is the ESBL required for the protective effect

The potential degradative effect of TEM, OXA and CTX-M-15 was studied using *E. coli* 33 monitored over 12 h in four different CM obtained from *E. coli* JM109 OXA (CM pGEM-OXA), JM109 TEM (CM pGEM-TEM), and JM 109 CTX-M-15 (CM pGEM-CTX-M-15). An experiment was done as control with *E. coli* JM109 containing pGEM-T with no insert (CM pGEM); in presence of cefotaxime strain 33 failed to proliferate (Figure 3.10).

The next experiments were conducted in absence or in presence of 8 µg/ml and 32 µg/ml of cefotaxime (Figure 3.10). In presence of CM from pGEM-OXA and CM from pGEM-TEM strain 33 was able to grow for approximatively 2 h before growth declined. In contrast strain 33 was able to proliferate in the presence of CM pGEM-CTX-M-15 at all concentrations of cefotaxime tested. This result showed that only CM pGEM-CTX-M-15 provided a protective effective by degradation of the cefotaxime.

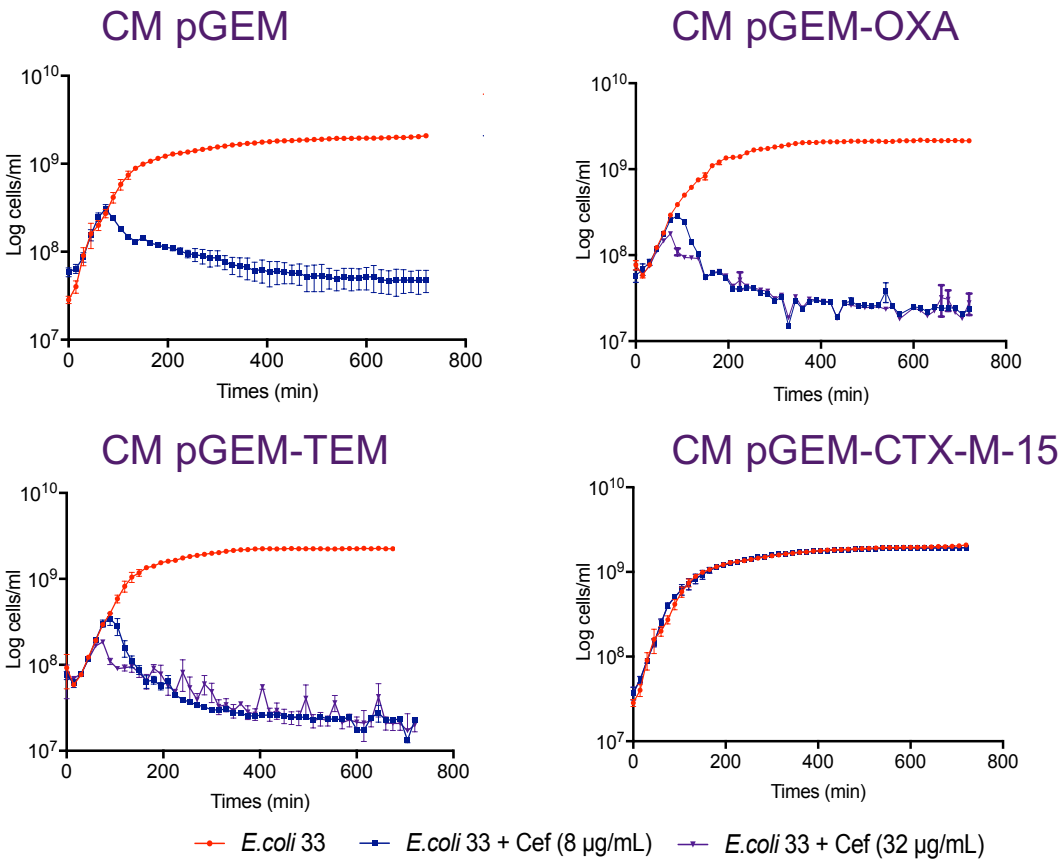


Figure 3.10 Growth of *E. coli* strain 33 cultivated in fresh LB media and different CM: CM pGEM, CM pGEM-OXA, CM pGEM-TEM, CM pGEM-CTX-M-15 in absence and presence of 8 and 32 µg/ml of cefotaxime.

3.3.6 Stability of CTX-M-15

We tested the stability of the CTX-M-15 enzyme by storing the CM obtained from JM109-CTX-M-15 at 4°C for 24 h, 48 h and 72 h, prior to inoculation with strain 33. Growth of strain 33 was monitored in the CM for a 12-hour period in presence of 8 µg/ml cefotaxime. Strain 33 was able to proliferate in all the media tested (Figure 3.11).

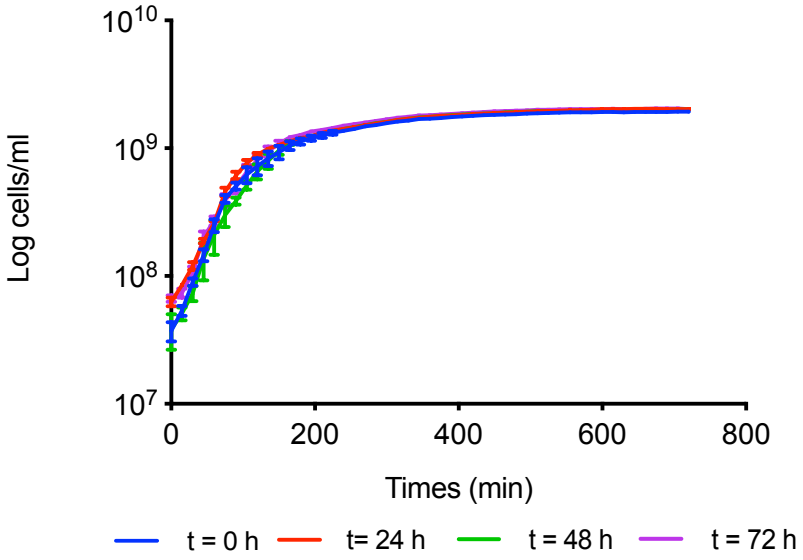


Figure 3.11 Growth of strain 33 cultivated in CM pGEM-CTX-M-15 in presence of 8 µg/ml of cefotaxime. Experiment was conducted with the CM pGEM-CTX-M-15 being at 4º C for 24 h, 48 h, and 72 h.

3.3.7 Secretion of CTX-M-15

3.3.7.1 Investigation of CTX-M in vesicles

CTX-M-15 was identified in the exoproteome and the enzyme had a degradative effect on cefotaxime and thus provided a protective effect to susceptible strains. In the scope of identifying a mechanism of secretion for this enzyme, we initially investigated the role of OMVs. To remove OMVs from the supernatant, CM obtained from the environmental strain 48 was filtered through a 0.02 µm filter. The ability of *E. coli* 33 to proliferate in the filtered CM and in presence of cefotaxime was investigated (Figure 3.12). In presence of all concentrations of cefotaxime tested, cells were able to proliferate. This suggests no role for OMVs in the secretion and extracellular release and stability of CTX-M-15.

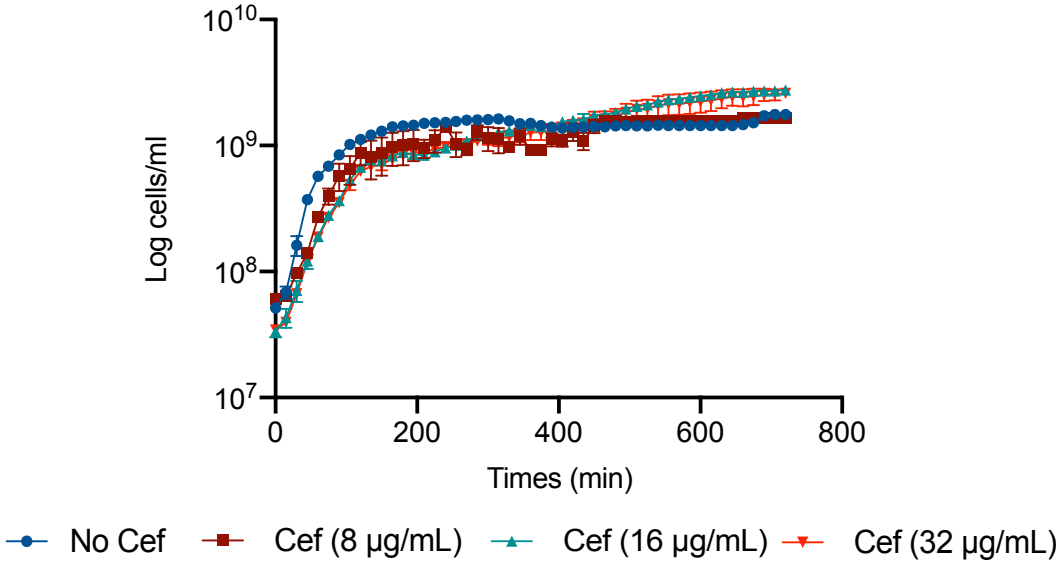


Figure 3.12 Growth of strain 33 cultivated in CM obtained from strain 48 after filtration of the CM through a 0.02 µm filter. Experiment was conducted in absence and presence of cefotaxime (8, 16 and 32 µg/ml).

3.3.7.2 *In silico* analysis

Given the fact OMVs did not seem to harbour the CTX-M-15, the mechanism for secretion across the outer-membrane remained unknown.

3.3.7.2.1 Signal peptide prediction

The SignalP showed the presence of signal peptide for both CTX-M-15 and TEM but not for OXA (Figure 3.13). CTX-M-15 and TEM were predicted to present a signal peptide with a D-score of 0.817 and 0.710 respectively (Table 3.6). The server also discriminated between the types of signal peptides, either cleaved by the Sec or the Tat pathway. Both CTX-M-15 and TEM were predicted to have a Sec signal peptide (Sec/SPI).

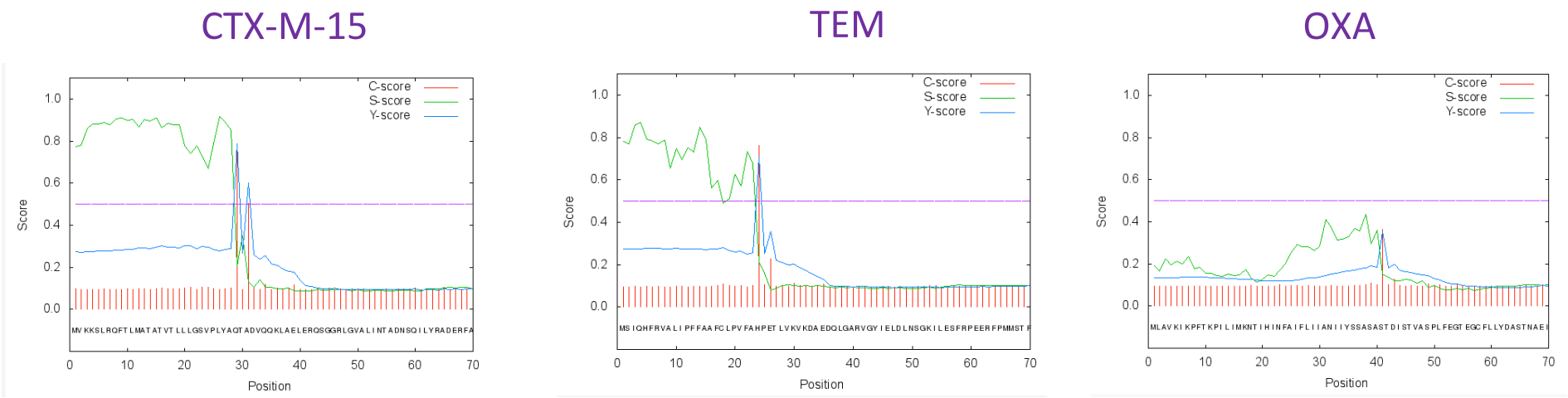


Figure 3.13 SignalP server predicted a signalling peptide for CTX-M-15 and TEM. The S score (green line) was used to predict the residues of the signal peptide. The cleavage site was determined by the C score (red line). The better cleavage site was determined by the Y score, which combines the predicting of the S and C scores.

Table 3.6 D-Score predicted by SignalP server.

	D-score (cut-off =0.570)
CTX-M-15	0.817
TEM	0.710
OXA	0.308

3.3.7.2.2 Secretion system type in *E. coli* prediction

Combining the exoproteomic and *in silico* analyses, it is likely that a specialised secretion system is involved in the translocation of CTX-M-15 across the outer membrane. In order to elucidate the mechanism of secretion of CTX-M-15, the different secretion systems in strain 48 must be identified. Using the Galaxy server, four different secretion systems were predicted; the T1SS, T2SS, T4SS and T5SS. As described in the introduction (see 3.1) T1SS and T2SS have the potential to be involved in the secretion of hydrolytic enzymes, such as ESBLs.

3.3.8 Investigating the secretory mechanism

3.3.8.1 Alternative host expression

As it was hypothesised that secretion of CTX-M-15 may involve the T1SS or the T2SS, mutagenesis or these two secretions system should result in a partial or total lack of CTX-M-15 secretion.

The pGEM-T-CTX-M-15 construct, shown to be essential for cefotaxime resistance (Section 3.3.5.2.2), was transformed into two different *E. coli* secretion system mutants: *E. coli* Δ*tolC* (T1SS KO), *E. coli* Δ*gspD* (T2SS KO), obtained from the Keio database. The wildtype parental strain of these mutant *E. coli* BW 25113 possessing intact secretion systems was used as a control.

Colony PCR showed the presence of the CTX-M-15 in the transformant. Successful transformant containing pGEM-CTX-M-15 was labelled *E. coli* BW+, *E. coli ΔtolC*+ and *E. coli ΔgspD*+.

3.3.8.2 Effects of *KO*

E. coli BW, *E. coli ΔtolC* and *E. coli ΔgspD* containing pGEM-t with no insert was used as a control. Proliferation was monitored over a period of 12 h period in absence and presence of 8 μg/ml of cefotaxime (Figure 3.14 A). In presence of cefotaxime *E. coli* BW, *E. coli ΔtolC* and *E. coli ΔgspD* failed to proliferate.

To investigate the effect of the KO of the T1SS and the T2SS on the growth of *E. coli* BW+, *E. coli ΔtolC*+ and *E. coli ΔgspD*+, the three strains were exposed to varying concentrations of cefotaxime and growth was monitored over 12 h (Figure 3.14 B).

The parental wild type *E. coli* BW+ was resistant to all concentrations tested. Surprisingly, the two other mutants, *E. coli ΔtolC*+ and *E. coli ΔgspD*+, also grew with all concentrations of cefotaxime. KO of the and T1SS and T2SS did not affect the growth in presence of cefotaxime. Exponential growth rate was calculated for *E. coli* BW+, *E. coli ΔtolC*+ and *E. coli ΔgspD*+ (Appendix 5).

Additionally, resistance to cefotaxime exhibited no fitness cost for cell division, as biomass reached in the presence of the antibiotic was similar to the one obtained without it.

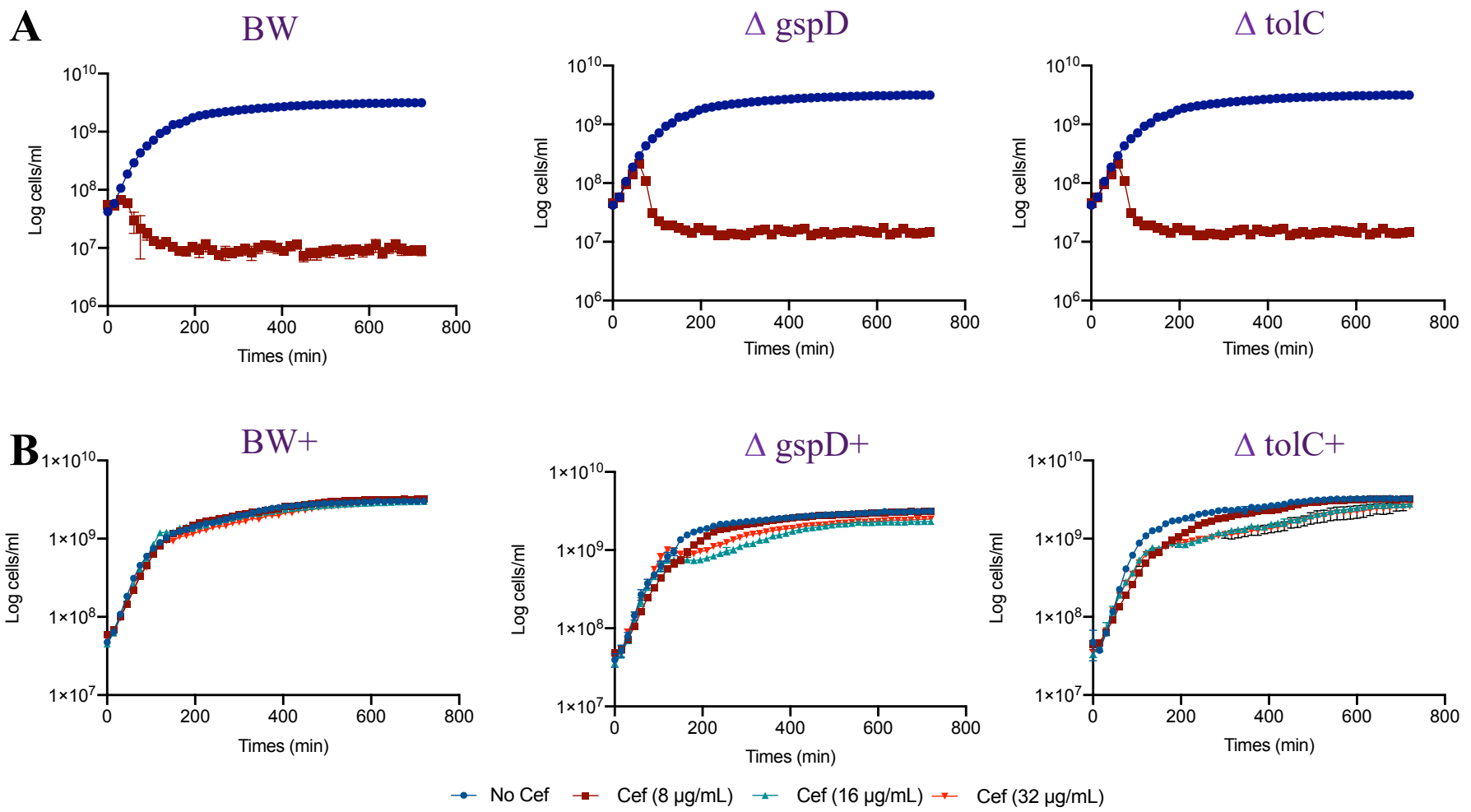


Figure 3.14 A) Growth of *E. coli* BW, *E. coli* Δ*gspD* and *E. coli* Δ*tolC* in absence and presence of cefotaxime (8 µg/ml). (**B**) Growth of *E. coli* BW+, *E. coli* Δ*gspD*+ and *E. coli* CM Δ*tolC*+in absence and presence of cefotaxime (8, 16 and 32 µg/ml).

3.3.8.3 Role of T1SS and T2SS for secretion of CTX-M-15

The previous experiment highlighted the ability of *E. coli ΔtolC*+ and *E. coli ΔgspD*+ to grow in presence of varying concentrations of cefotaxime. Although the growth was still possible in these two KO mutants, the presence of CTX-M-15 in the exoproteomic part of *E. coli ΔtolC*+ and *E. coli ΔgspD*+ was not demonstrated. To address this question, the proliferation of strain 33 was monitored over 12 h in presence of CM obtained from the three previous constructs (CM *E. coli* BW+, CM Δ*tolC*+, CM Δ*gspD*+), as previously performed.

Strain 33 proliferated from ~3.10^7 cells/ml to 3.10^9 cells/ml in the three different conditioned media tested: CM *E. coli* BW+, CM Δ*tolC*+, CM Δ*gspD*+ at all concentrations of cefotaxime (Figure 3.15). Growth rate were calculated for strain 33 in CM Δ*gspD*+ and CM Δ*tolC*+ and showed a significantly different rate in CM Δ*gspD*+ and CM Δ*tolC*+ in presence of 8 µg/ml (Appendix 6).

Taken together, these data provide evidence for β-lactamase secretion into the surrounding environment. The CTX-M-15 provided a protective effect to susceptible strain 33. CTX-M-15 was still secreted in the mutant with KO T1SS and T2SS.

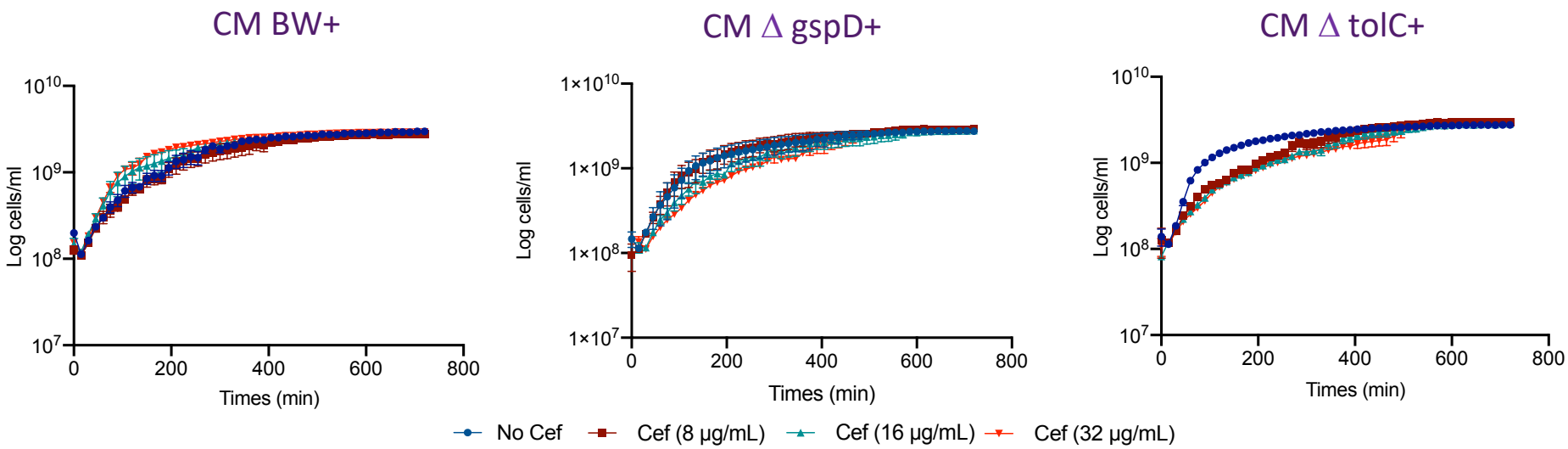


Figure 3.15 Growth of Strain 33 cultivated in different CM obtained from *E. coli* BW+ (CM BW+), *E. coli* Δ*gspD*+ (CM Δ*gspD*+), and *E. coli* CM Δ*tolC*+ (CM Δ*tolC*+) in absence and presence of cefotaxime (8, 16, 32 µg/ml).

3.4 Discussion

Whilst strain 48 possesses three annotated ESBLs, the data presented in this Chapter clearly shows that CTX-M-15 is the major secreted ESBL conferring resistance to strain 48. Furthermore, through enzymatic assays, comparative proteomics, and cultivation experiments, it was also revealed that this enzyme is secreted outside of the cell into the extracellular milieu. This is similar to previous studies but for the first time we show that CTX-M-15 is also secreted in this manner (Kim *et al.*, 2018).

Peptide identification using LC-MS/MS established CTX-M-15 as the third most abundant protein in the exoproteome. Comparison between the whole-cell proteome and exoproteome allowed detection between secreted and leaked cytoplasmic protein and possible lysis products. The relative abundance of CTX-M-15 (2.48 %) in the exoproteome was 10-fold greater than in the cytoplasmic extract (0.27 %) derived from lysing whole cells, the data clearly demonstrates CTX-M-15 is actively secreted outside the cell. The osmotically inducible protein OsmY was the most abundant protein found in the exoproteomic part (4.08 %) which was 15-fold greater than its abundance in the cytoplasm (0.27 %). A previous study showed that this protein was naturally secreted by *E. coli* laboratory strains, *E. coli* Top 10 and *E. coli* BL21 (Qian *et al.*, 2008). Therefore, OsmY is often used in biotechnology to deliver proteins into the medium by fusion with the C-termini of protein (Bokinsky *et al.*, 2011). OsmY possesses a signal peptide sequence predicted to be targeted for secretion into the periplasm *via* the Sec pathway, where it is then folded and secreted into the medium (Qian *et al.*, 2008). This agrees with the finding of the current study where signal peptide of both OsmY and CTX-M15 were both SecA type for directing proteins to the periplasm. The mechanisms responsible for OsmY secretion has not been resolved, however both the *E. coli* strain used in Qian *et al*. (2008) and the strain used in this Chapter had the T2SS (Qian *et al.*, 2008). Initially this secretion system was suspected of being responsible for excretion of CTX-M-15 into the exoproteome in the current study.

Bioinformatic tools also allow discrimination between cytoplasmic and exoproteomic protein and the combination of predictive tools with proteomic data has been shown to be effective for *E. coli* (Han et al., 2011). *In silico* analysis predicted a signal peptide

and a non-classical secretion for CTX-M-15. Reports concerning secretion in *E. coli* remain elusive mainly because non-pathogenic laboratory strains generally express a small amount of proteins in the culture medium (Papanikou *et al.*, 2007; Kotzsch *et al.*, 2010).

Growth of both the T1SS (Δ*tolC*) and T2SS (Δ*gspD*) mutant strains containing pGEM-CTX-M-15 in the presence of cefotaxime still occurred. Furthermore, the protective effect of CTX-M-15 secretion was still present. It may be that a double KO is needed to ensure both secretion systems do not act co-operatively.

Whilst packaging of β-lactamases into OMVs has been demonstrated in *Pseudomonas aeruginosa* an in *E. coli* DH5 α for the secretion of NDM-1 (Ciofu *et al.*, 2000; Gonzalez *et al.*, 2016), OMVs did not appear to be involved in the secretion of CTX-M-15. NDM-1 is the most widespread carbapenemase worldwide and is an MBL and has a lipobox proximal to its signal peptide. The stability of NDM-1 was due to the lipidation of the enzyme allowing anchoring to the outer-membrane of the *E. coli* host and subsequent export in OMVs (Gonzalez *et al.*, 2016). Removal of the lipobox inhibited the export of the enzyme *via* OMVs and the enzyme accumulated in the periplasm. OMVs containing NDM-1 present evolutionary advantages including protection of bacteria located in the surrounding environment which select for survival of resistant and non-resistant bacterial and protection of the enzyme by proteases. NDM-1, BRO-1 (from the human pathogen *Moraxella catarrhalis*) and PenA (from *Burkholderia pseudomallei*) are the only lipidated β-lactamases that have been reported in Gram-negative bacteria (Bootsma *et al.*, 1996; Randall *et al.*, 2015). In agreeance with a lack of OMV involvement, no lipobox was predicted using the signal P software in CTX-M-15.

Kim *et al.* (2018) implicated OMVs in the secretion of a serine β-lactamase CTX-M-1 in OMVs secreted by *E.* coli host of uncertain genotype referred to a RC85 (Kim *et al.*, 2018). They reported a method for recovery of OMVs and proved that the vesicular fraction could provide protective effect to sensitive strains for cefotaxime, ampicillin and cefoperazone. Proteomic analysis of the vesicular fraction revealed the presence of CTX-M-1, as well as various membrane proteins, such as outer-membrane porins in addition to periplasmic binding proteins. However, careful analysis of this data suggests that cytoplasmic leakage may have also occurred because OMV fraction

contains a wide range of proteins found in the cytoplasm which would not be expected in OMVs derived from the periplasm. These included ribosomal proteins and cytoplasmic proteins. Attempts were made to investigate OMV presence in strain 48 but very few vesicles were recovered. Filtration of the conditioned medium obtained through a 0.02 μm membrane still provided the protective effect.

Despite being unable to clearly demonstrate a secretory mechanism for strain 48, this Chapter has demonstrated that CTX-M-15 is the major secretory ESBL which can confer a protective effect to neighbouring susceptible cells.

Chapter 4:

Investigating the fate of *E. coli* ST131 in an anaerobic bioreactor

4.1 Introduction and aims

Anaerobic digestion (AD) provides a solution to reducing agricultural waste leading to improved sanitation and a reduction in greenhouse gas emissions through production of usable biogas (methane) for energy and high-quality nutrient rich, odour free fertilizer. The digestion process results in the degradation of complex organic materials (animal manure, sewage sludge) to simpler ones in the absence of oxygen *via* the action of metabolically distinct microorganisms in hydrolytic, acidogenic, acetogenic, and methanogenic settings (Manyi-Loh *et al.*, 2013). The digester is influenced by temperature and the reaction can occur in three ranges: psychrophilic (0ºC to 20ºC), mesophilic (20ºC to 45ºC) and thermophilic (45ºC to 75ºC). Theoretically the process presents an attractive solution to the disposal of agricultural waste/animal manure, however there are concerns over the selective pressures that are induced in anaerobic bioreactors (ABs): it is suggested antibiotics might result in ARG dissemination and sub-lethal concentrations induce the oxidative stress response resulting in mutagenesis and an increase in multi-resistant bacteria (Ghosh *et al.*, 2009; Kohanski *et al.*, 2010). Several studies investigated levels of biocide in manure and reported high levels of tetracycline and heavy metals which could select for resistance (Pan *et al.*, 2011; Qiao *et al.*, 2012; Zhu *et al.*, 2013). Research has highlighted the importance of the activated sludge (AS) from the WWTPs in the dissemination of ARGs and the AS is regarded as a hotspot for HGT and ARGs due to its bacterial load with high levels of faecal bacteria (Kümmerer and Henninger, 2003; Gaze *et al.*, 2011). The degradation of antibiotics is primarily an aerobic process and most drugs do not undergo significant breakdown during water treatment.

According to a DEFRA report in 2010, 18.8% of sewage sludge was incinerated, 0.6% put to landfill, 0.2% was disposed of through other methods, 1.7% was redistributed for unspecified uses and the remaining 79.1% of sewage sludge was re-used for agricultural purposes. The concentration of antibiotics within the sludge varies,

depending on the origin of the influent, the presence of hospital and the type of the plant (secondary or tertiary) and its operational parameters (mesophilic, thermophilic) (Chen *et al.*, 2013). The substantial amount of sewage that is applied to land is contributing to the environmental resistome with large numbers of ARGs found in soils treated with sludge (Smalla *et al.*, 2000; Heuer *et al.*, 2012).One study reported that more than 700 genera and 3000 operational taxonomic units (OTUs) have been identified in the AS (Zhang *et al.*, 2011). A study investigated plasmids recovered from the AS derived from a WWTP in China reported ARGs from a diverse range of antimicrobial classes and MGEs present in the plasmid metagenome (Zhang *et al.*, 2011). The addition of nutrient rich manure to soil is shown to have a substantial impact on the abundance and mobility of ARGs, with repeated application of manure increasing resistance gene prevalence and as bacteria from manure may not be well adapted to the soil environment, ARGs could be horizontally transfer to soil bacteria (Heuer *et al.*, 2011; Heuer *et al.*, 2012).

There is growing interest in the potential to limit the spread of ARGs, in particular, operational conditions during sludge digestion may serve to discourage selection of ARB, reduce HGT and aid in hydrolysis DNA (Xue *et al.*, 2019; He *et al.*, 2020).

Whilst AD appears to be a major improvement in manure disposal and pathogen reduction, there is a clear risk that it may not eliminate ARGs and may in fact result in the persistence and wider dissemination *via* recycling of waste material on the farm following digestion. The *E. coli* isolates “48” and “129” were selected from the strain bank of *E. coli* isolated form the River Sowe (UK) (Amos *et al.*, 2014; Hill, 2016; Amos *et al.*, 2018). The aim of this study was to examine the effectiveness of AD on $bla_{\text{CTX-M-15}}$ gene prevalence, pathogen removal and characterisation of the microbial community present in the bioreactor. The impact of sub-lethal concentration of cefotaxime, a third-generation cephalosporin, on $bla_{\text{CTX-M-15}}$ was investigated using small-scale *in-vitro* bioreactors. Cefotaxime is used in the treatment of mastitis and foot disease in cattle and the drug may last for several days during AD, there have been reports of both selection and co-selection of ARGs in bioreactors containing biocides and /or antibiotics (Kromker and Leimbach, 2017).

4.2 Material and methods

4.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 4.1. *E. coli* strains were isolated from the River Sowe, Coventry, UK; with recovery of *E. coli* ST131 O25:H4 (strain 48) and *E. coli* ST3574 O105:H20 (strain 129) (Hill, 2016). Resistance and phenotype profile are listed in Table 4.1 and Table 4.2.

Table 4.1 Bacterial strains and plasmids used in this study

Strain	Genotype and comments	Reference
E. coli 48	*bla*$_{CTX-M-15}$, *bla*$_{TEM}$, *bla*$_{OXA}$, *sul1, vanG*	This study
E. coli 129	/	This study

Table 4.2 Phenotypic resistance profile of strains 48 and 129

Isolate	Ampicillin (25 µg	Cefotaxime (5 µg)	Imipenem (10 µg)	Chloramphenicol (30 µg)	Erythromycin (8 µg)
48	Yes	Yes	No	No	Yes
129	No	No	No	No	No

Cells were routinely grown in LB liquid broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or LB solid (addition of 15 g/L agar) medium, supplemented with 8 µg/ml of cefotaxime when required. Cells were incubated at 37ºC with either shaking (200 rpm) or static conditions. All *E. coli* strains were stored in 8% (v/v) glycerol at -80ºC.

4.2.2 Biomass collection

Digestate was provided by Stephen Nolan (National University of Ireland, Galway) and were obtained from a continuously stirred tank reactor (CSTR) operated at 37ºC used for the co-digestion of dairy cattle slurry (DCS) and fats, oils, grease (FOG) at a ratio of 2:1 for more than a year. 2.5 L were sampled 3 days (d) after a batch of slurry was introduced to the digester, kept at 4ºC and mixed thoroughly before processing to the experiment.

4.2.3 Microcosms

Microcosms were prepared in 120 ml glass bottles containing 100 ml of biomass. Three conditions, in triplicate were tested; *E. coli* 48 amended (R), *E. coli* 129 amended (S) and unamended (C). Strain 48 and 129 were spiked in order to have a starting concentration in the microcosm of 10^5 CFU/ml. The experiment was performed in absence or in presence of 8 μg/ml cefotaxime. All microcosms were sealed sparged with N_2 gas before incubation at 37ºC with shaking (200 rpm) for 9 days.

Antibiotic were refreshed daily to have a final concentration of 8 μg/ml of cefotaxime. Every three days, 12 ml aliquot of the biomass were collected from each microcosm and snap-frozen using liquid nitrogen and store at -80ºC until DNA extraction was performed.

4.2.4 Biogas measurement

The overpressure from the headspace of each microcosm were withdraw and measured each day by using a needle and syringe to give a biogas volume in ml, no additional headspace was sampled.

4.2.5 Methane measurement

The methane content (%) in the biogas headspace was measured by gas chromatography using the CP-3800 (VARIAN Inc.) equipped with a glass column and a flame ionization detector. Nitrogen was used as the gas carrier with a constant flow rate of 30 $ml.min^{-1}$. Methane (100%) was used as a standard for the chromatography calibration and the data were recorded through the Varian Start Chromatography integration software.

4.2.6 Determination of the background *E. coli* in the biomass

E. coli and total coliforms were quantified using Colisure with Quanti-Tray 2000® (IDEXX) following the manufacturer's instructions. Biomass was diluted as necessary to fall within the detection range by using phosphate buffer saline (PBS). Positive

wells were counted, and the Most Probable Number (MPN) were obtained. The results were interpreted as followed; yellow/gold wells are negative for total coliform and *E. coli,* red or magenta positive for total coliforms and red/magenta and fluorescence at 364 nm positive for *E. coli*. The detection limit is 1 MPN per 100 ml of sample.

4.2.7 DNA extraction

DNA was extracted using the DNeasy® PowerSoil® Kit (Qiagen) power kit following the manufacturer's instructions and resuspended in a final volume of 50 µl and stored at -20ºC.

4.2.8 DNA quantification

DNA was quantified using the Qubit® fluorometer (Invitrogen). The Qubit dsDNA Broad Range BR kit (Invitrogen) was used according to the manufacturer's instructions.

4.2.9 qPCR

The 7500 Fast Real-Time PCR (Applied Biosystem) was used to perform all the qPCR assays which were set up using 12.5 µl of environmental Taqman™ (Fisher Scientific), 0.4 mg/ml BSA, 0.4 µM forward primer, 0.4 µM reverse primer, 2 µM probe, 1 µl DNA template made up to 25 µl using DNA free water. A list of primers and probes used are listed in Table 4.3. The $bla_{\text{CTX-M}}$ Group 1 targets the $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$.

qPCR conditions for each reaction were as followed: 95ºC for 10 mins, 40 cycles of 95ºC for 15 s, 60ºC for 30 s. and then 95ºC for 60 secs.

Table 4.3 Primers and probes used for qPCR.

Target	Primer name	Sequence	Probe	Product size	Reference
16S rRNA	1369F	5'CGGTGAATACGTTCYCGGGG3'	6JOE-CTTgTACACACCgCCCgTC--BBQ	124	(Czekalski *et al.*, 2014)
	1492R	5'GGWTACCTTGTTACGACT3'			
bla$_{CTX-M}$ Group 1	CTX-F	5'ATGTGCAGYACCAGTAARGTKATGATGGC3'	5-Joe-CCCGACAGCTGGGAGACGAAACGT-Tamra	85	(Birkett *et al.*, 2007)
	CTX-R	5'ATCACKCGGRTCGCCXGGRAT3'			

qPCR standards for the quantification of all the genes tested were ordered from IDT using the gBlocks® Gene Fragments technology for the synthesis (Table 4.4). qPCR standard curves were created amplifying serial dilutions of the synthetized oligo using six point (from 10^7 to 10^2 copies) measured in triplicate.

Table 4.4 Oligos used as standards

Gene	Oligo
16S rRNA	AACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG CCCGTCAAGTCATGAAAGTGGGTAGCACCCGAAGCCGGTG GCCCGACCCTCGTGGGGGGAGCCGTCTAAGGTGAGACTCG TGATTGGGACTAAGTCGTCGTAACAAGGTAGCCGTACCAA

4.2.10 Normalisation of qPCR

16S rRNA gene quantity was then normalized by 2.5 as this is the average 16S rRNA gene copies in general bacteria according to the copy number reported on the ribosomal RNA database (rrnDB-4.4.4 – https://rrndb.ummms.med.umich.edu). Quantified data were then Log10 transformed to normalize the qPCR count.

4.2.11 16S rRNA gene amplicon sequencing

DNA was quantified and normalised at 5 ng. 16S rRNA gene amplicon was carried as specified in Illumina Miseq guide for 16S metagenomics library preparation (Illumina). The V3-V4 region of the 16S rRNA gene of bacteria was amplified using the forward primer CCTACGGGNGGCWGCAG and reverse primer GACTACHVGGGTATCTAATCC. PCR amplifications were performed using Q5®

Hot Start High-Fidelity 2X Master Mix (New England Biolabs), using PCR conditions as followed; initial denaturation at 95ºC for 3 min, followed by 25 cycles of denaturation at 95ºC for 30 sec, annealing temperature at 55ºC for 30 sec and extension at 72ºC 30 sec. A final extension was performed at 72ºC for 5 mins. PCR clean-up was done using the AMPure XP beads (Beckman Coulter). Index PCR was performed using Illumina Nextera Index Kit v2 adapters. Sample normalization was done with the SequelPrep™ Normalisation Plate Kit (ThermoFisher Scientific) and samples were pooled for sequencing. Libraries were pooled, quantified using the Qubit fluorometer (Invitrogen) with the Qubit dsDNA Broad Range BR kit (Invitrogen) and diluted to 4 nM, and then denatured and loaded onto the flowcell of the Miseq.
PCR was performed at an initial denaturation temperature at 95ºC for 3 min, followed by 25 cycles of denaturation at 95ºC for 30 sec, annealing temperature at 55ºC for 30 sec and extension at 72ºC 30 sec.

4.2.12 Data analysis of 16S rRNA gene amplicon

Sequence processing was performed in RStudio v1.2.1335 and DADA2 package was used to obtain the amplicon sequence variants (ASVs) (Callahan *et al.*, 2016; Callahan *et al.*, 2017). 16S rRNA gene forward and reverse primers sequences were trimmed at 17 bp and 21 bp respectively, chimeras were removed and taxonomy was assigned using IDTAXA implemented in the R package DECIPHER with the SILVA v132 database (Wright, 2016; Murali *et al.*, 2018). The algorithm Vsearch was used to convert ASVs to operational taxonomic units (OTUs) with 97% similarity (Rognes *et al.*, 2016). Sequencing coverage was inspected *via* rarefying curve. The alpha diversity of the communities was analysed on rarefied OTUs communities by calculating indices for Shannon diversity, Simpson and Simpson inverse. The Shannon and Simpson inverse take into account the richness and evenness of the species present in the sample to calculate the diversity; the higher the index is, the more diverse is the sample. Impact of time, spiking and antibiotic exposure on samples structuration was investigated through the use of an ordination methods, specifically non-metric multidimensional scaling (NMDS). This method needs a pairwise distance between sample for which the Bray-Curtis dissimilarity index was used. It allows to take into account both OTU shared presence or absence as well as their levels. NMDS observations were further inspected by the use of statistical testing regarding

aforementioned features. A PERMANOVA analysis was run through the adonis function of the R vegan library.

Data analysis, statistics and plotting were done using the following packages; phyloseq, multcomp, vegan and ggplot2 (McMurdie and Holmes, 2013).

4.3 Results

4.3.1 Slurry characterization

In the slurry sample the background *E. coli* count was 2 x 10^1 cell/ml. Bacterial abundance was determined, the total count did not change over the course of the experiment (Figure 4.1).

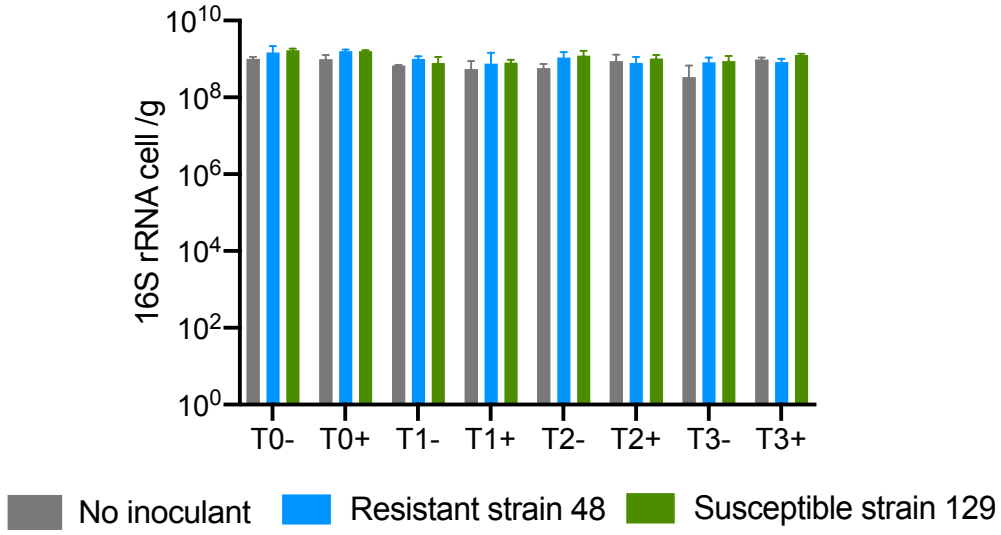


Figure 4.1 Abundance of 16S rRNA cell number per g in each sample over the 4 times points. With +: presence of cefotaxime; -: absence of cefotaxime, T1= sampling after 3 d; T2: sampling after 6 d and T3: sampling after 9 d.

4.3.2 Biogas production

The overpressure from the headspace of each microcosm was withdrawn daily and the accumulated biogas production was measured (Figure 4.2 **A**). Gas chromatography showed the production of CH_4 in each microcosm at day 3 (Figure 4.2 **B**).

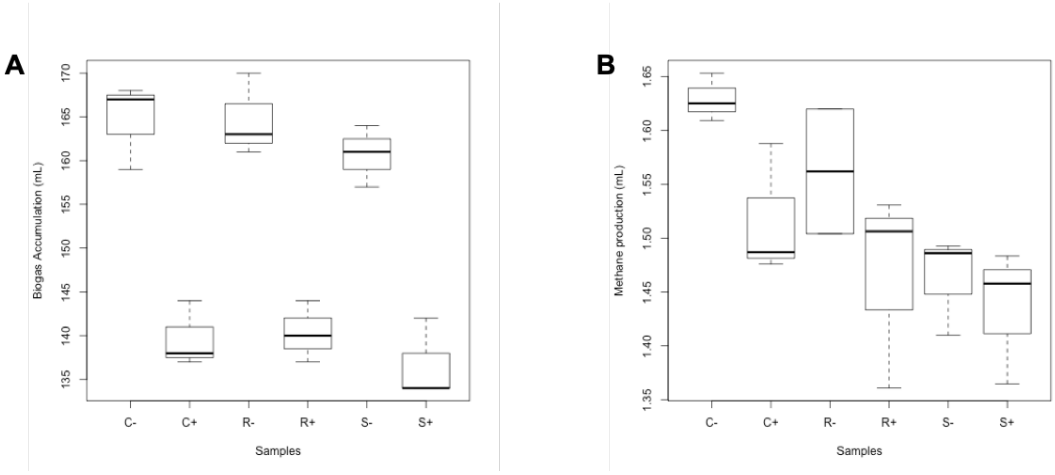


Figure 4.2 (**A)** Cumulative headspace biogas volume over the 9 days experiment. **(B)** CH_4 content of the biogas measured on the day 3 of the experiment. With C: biomass no amended; R: biomass amended with strain 48 and S: biomass amended with strain 129, +: presence of cefotaxime; -: absence of cefotaxime.

4.3.3 AD microbial community analysis

4.3.3.1 Rarefaction curves

All the rarefaction curves for the observed OTUs for each sample did not reach a plateau meaning that part of the diversity within the microbial community was not measured (Figure 4.3). Rarefaction was used to standardise all samples to the same number of reads for comparative purposes.

The rarefaction cut-off was done at 15,970 reads and excluded 10 samples below this level (Table 4.5).

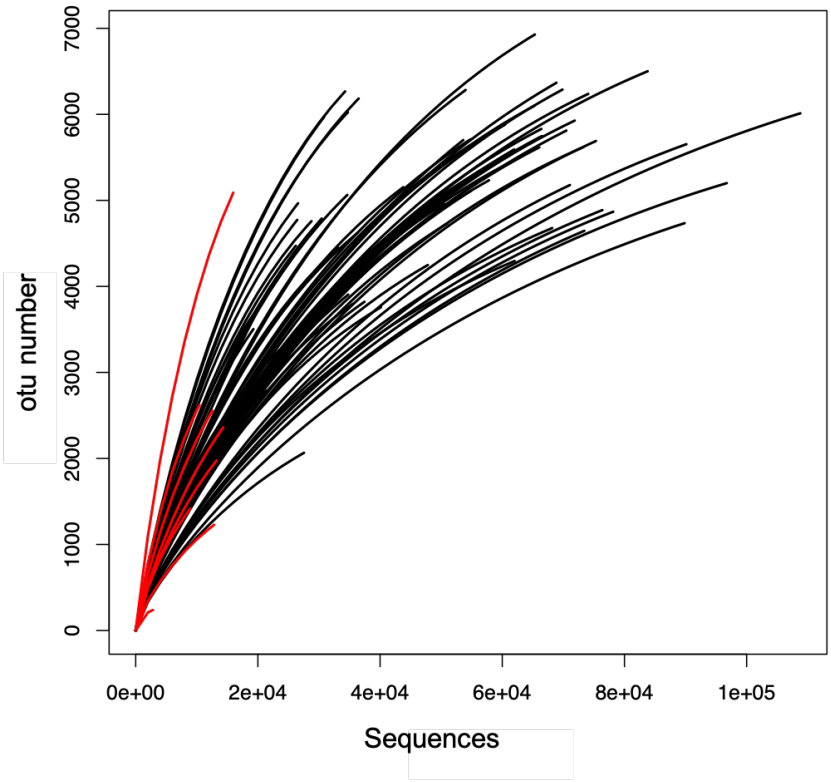


Figure 4.3 Rarefaction curves for 16S rRNA gene amplicon reads based on observed OTUs. The 72 samples are represented, red lines indicate samples discarded from the analysis.

Table 4.5 The ten samples with insufficient number of reads.

Treatment	Time Point	Cefotaxime	Reads per Sample
C	T0	No	12,831
C	T2	No	83
C	T2	Yes	14,311
C	T3	No	493
S	T0	No	2,823
S	T2	No	15,968
S	T3	Yes	12,558
R	T3	Yes	13,246
R	T1	No	10,445
R	T1	No	8,944

4.3.3.2 Identification of the microbial communities

Microbial community composition was investigated at different taxonomic levels. At a phylum level, the most abundant group was the Firmicutes followed by the Bacteroidetes (Figure 4.4). Euryarchaeota were recovered in most samples but may be underrepresented due to the use of the general 16S rRNA gene primers. Microbial communities at the order, family and genus level are respectively represented in Appendices 7, 8 and 9.

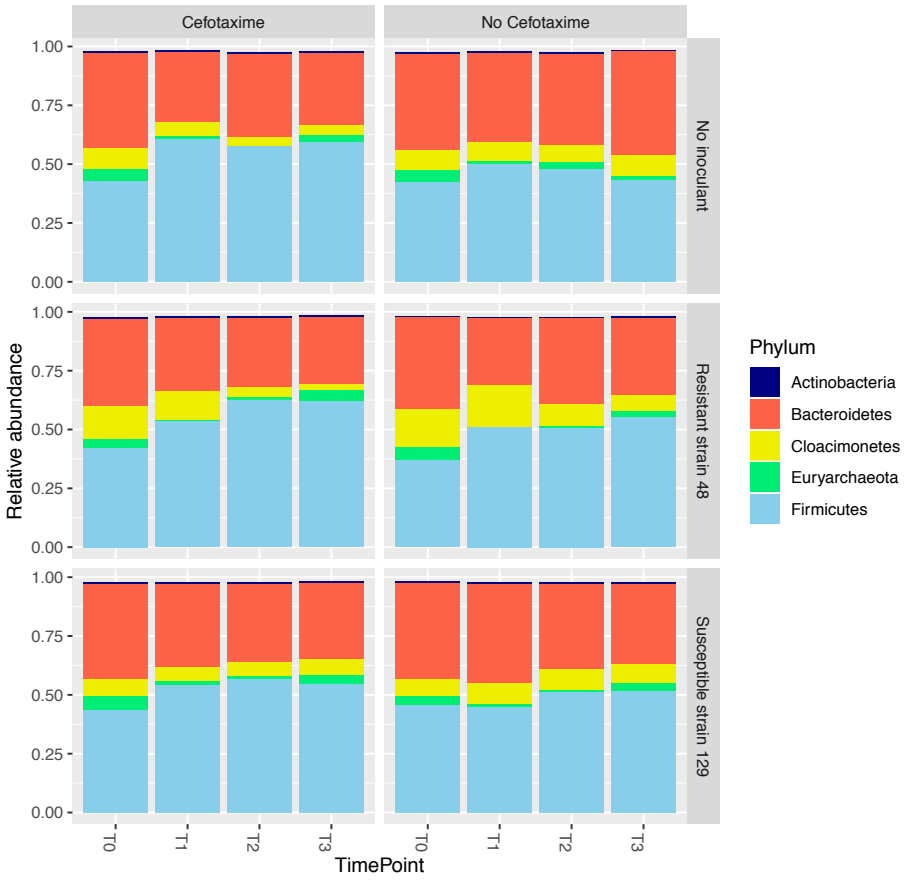


Figure 4.4 The dominant phyla for each sample at the four times points tested based on 16S rRNA gene annotation using Silva. Selected Archaea were also represented. Firmicutes and Bacteriodetes are the two main phyla.

4.3.3.3 Alpha and beta diversity analysis

An Adonis test was performed to determine if the treatments of spiking with *E. coli* and antibiotic addition had an impact on bioreactor bacterial community (Table 4.6). Analysis showed that 17% of the variance was attributed to cefotaxime impact (Adonis, R^2=0.17, p=0.001) on the community diversity at the genus level. The presence of the inoculants did not significantly impact the sample diversity (Adonis, R^2=0.03, p=0.079).

Table 4.6 Statistical summary of the Adonis test at the genus level on the 16S rRNA.

	Df	Sum Sqs	Mean Sqs	F.Model	R^2	P(>F)
Time point	3	0.59204	0.19735	11.3914	0.28180	0.001
Antibiotic	1	0.36870	0.36870	21.2824	0.17549	0.001
Treatment	2	0.06611	0.03306	1.9081	0.03147	0.079
Residuals	62	1.07410	0.01732		0.51124	

The alpha diversity was greater without cefotaxime and showed a greater variance across the incubation (Figure 4.5). Beta diversity analysis based on Bray-Curtis dissimilarity confirmed this pattern and identified two main groups related to treatment with the antibiotic (Figure 4.6).

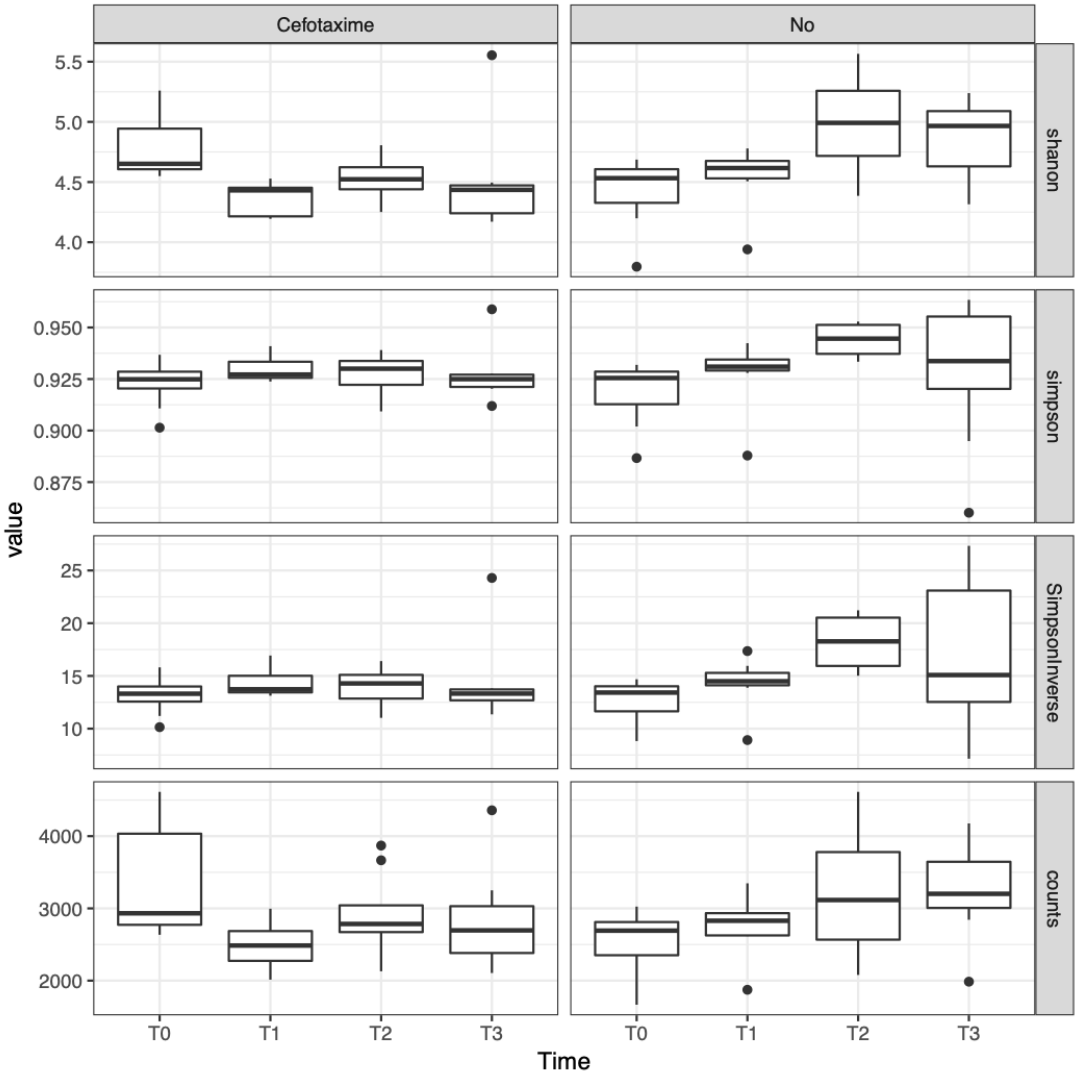


Figure 4.5 Analysis of the alpha diversity of the bacterial communities (16S rRNA gene) according to the presence of absence of antibiotics at the different time point

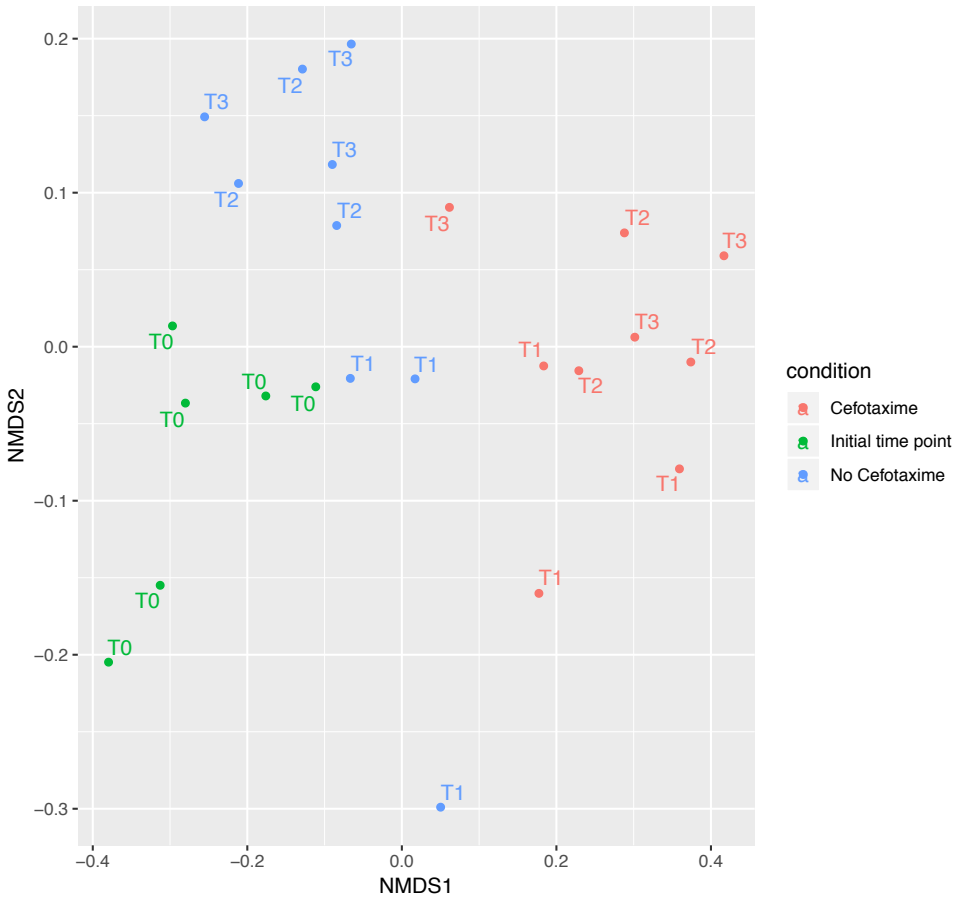


Figure 4.6 Nonmetric multidimensional scaling (NMDS) plots of bacterial communities (16S rRNA gene) based on Bray-Curtis dissimilarity indicating separation in presence or absence of cefotaxime in the microcosms.

4.3.3.4 Effect of the antibiotic at the genus level

The abundance of 11 genera was significantly impacted by the exposure to cefotaxime as illustrated in the volcano plot (Figure 4.7).

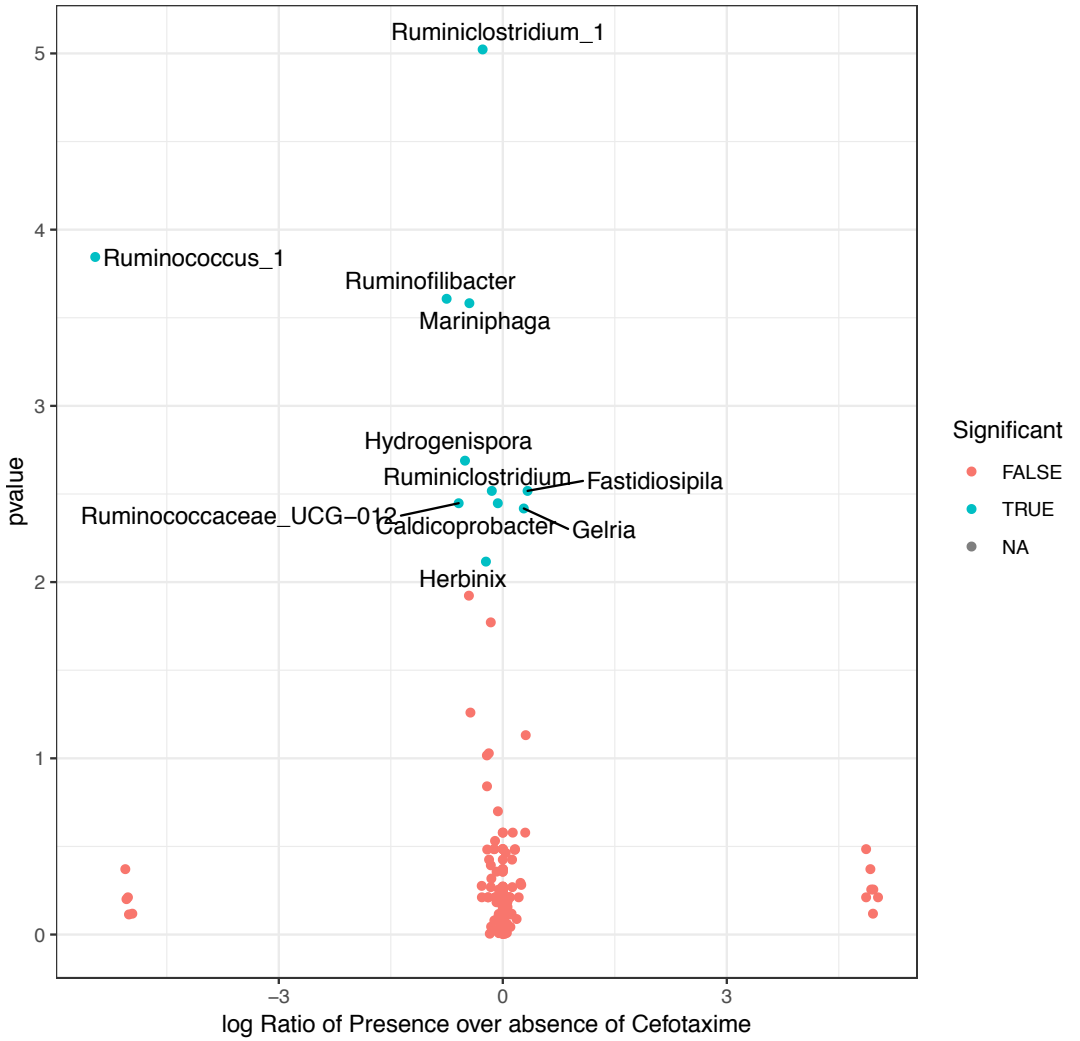


Figure 4.7 The impact of antibiotic on microbial community diversity. Scatterplot showing the statistical significance (-Log_{10}(Pvalue)) versus the fold change (ratio count in presence of cefotaxime over the count in absence of cefotaxime). The median value was used to calculate the ratio. Genus significantly impacted by the presence of cefotaxime in the microcosm are above 2 (pvalue <0.01). "True" means the genera is significantly impacted by the presence of cefotaxime.

The genera *Gelria* and *Fastidiosipila* were enriched in presence of cefotaxime and *Ruminclostridum_1*, *Ruminiclostridum*, *Mariniphaga*, Ruminococcaceae_UCG-012, *Herbinix*, *Caldicopracter*, *Ruminofilibacter*, *Ruminococcus_1* and *Hydrogenispora* were negatively impacted by the presence of cefotaxime (Figure 4.8) (Table 4.7).

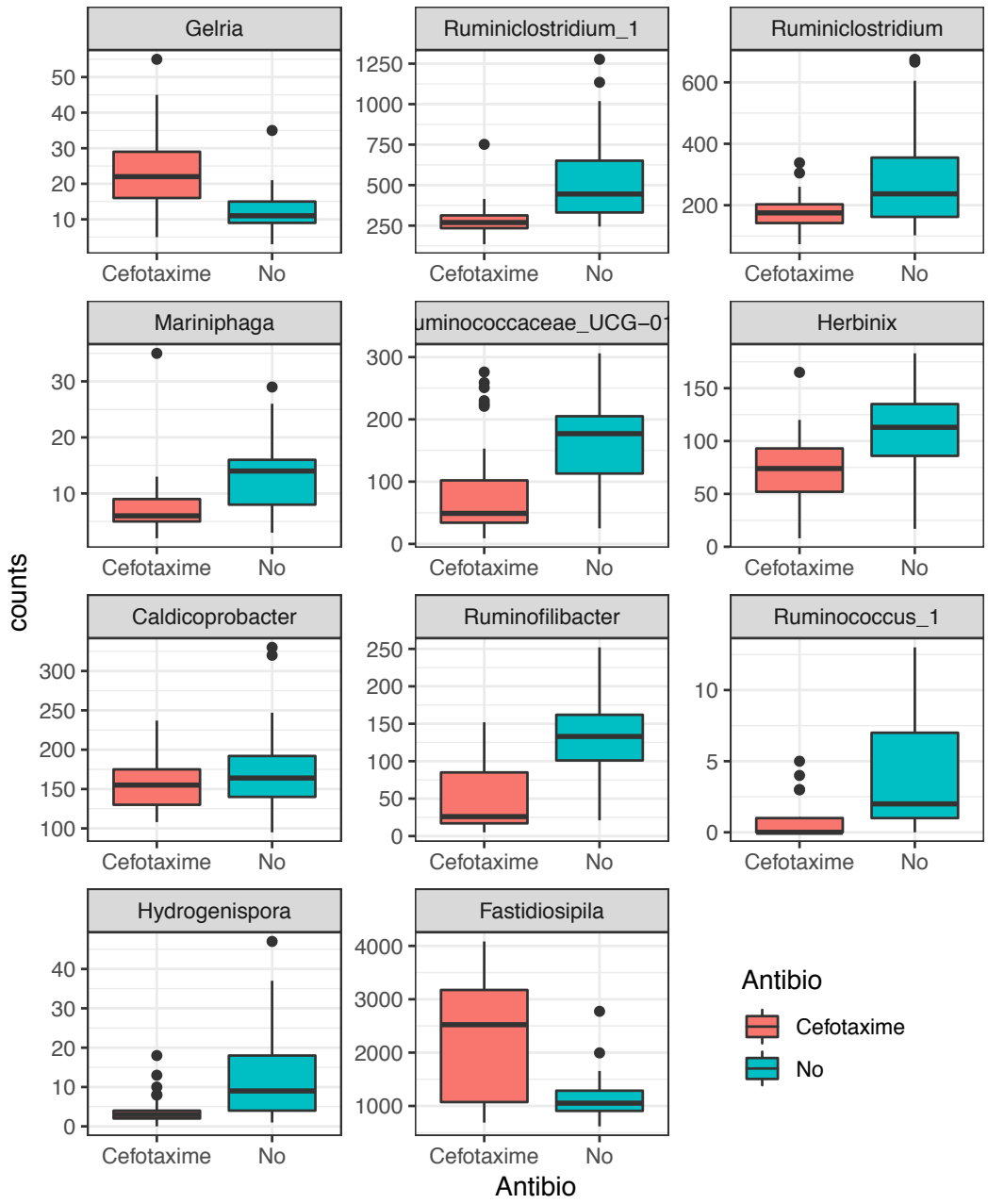


Figure 4.8 Genera impacted by the presence/absence of cefotaxime, abundance data for each taxon is represented by the count of each genera.

Table 4.7 The fold change in presence or absence of cefotaxime was significant with a p-value <0.01(Kruskal-Wallis).

Genus	Counts		P(>F)
	Cefotaxime	No Cefotaxime	
Ruminoclostridum_1	269	445	9.49×10^{-6}
Ruminococcus_1	0	2	1.43×10^{-4}
Ruminofilibacter	26	133	2.47×10^{-4}
Mariniphaga	6	14	2.61×10^{-4}
Hydrogenispora	3	9	2.05×10^{-3}
Fastidiosipila	2523	1052	3.04×10^{-3}
Ruminiclostridium	175	237	3.04×10^{-3}
Caldicoprobacter	155	164	3.57×10^{-3}
Ruminococcaceae_UCG-012	49	177	3.57×10^{-3}
Gelria	22	11	3.82×10^{-3}
Herbinix	74	113	7.65×10^{-3}

4.3.3.5 Fate of the inoculants

The microbial community analysed at the genus level revealed that Escherichia were generally absent or at very low levels in the control slurry whereas the spiked strain 48 appeared to persist over the incubation (Figure 4.9).

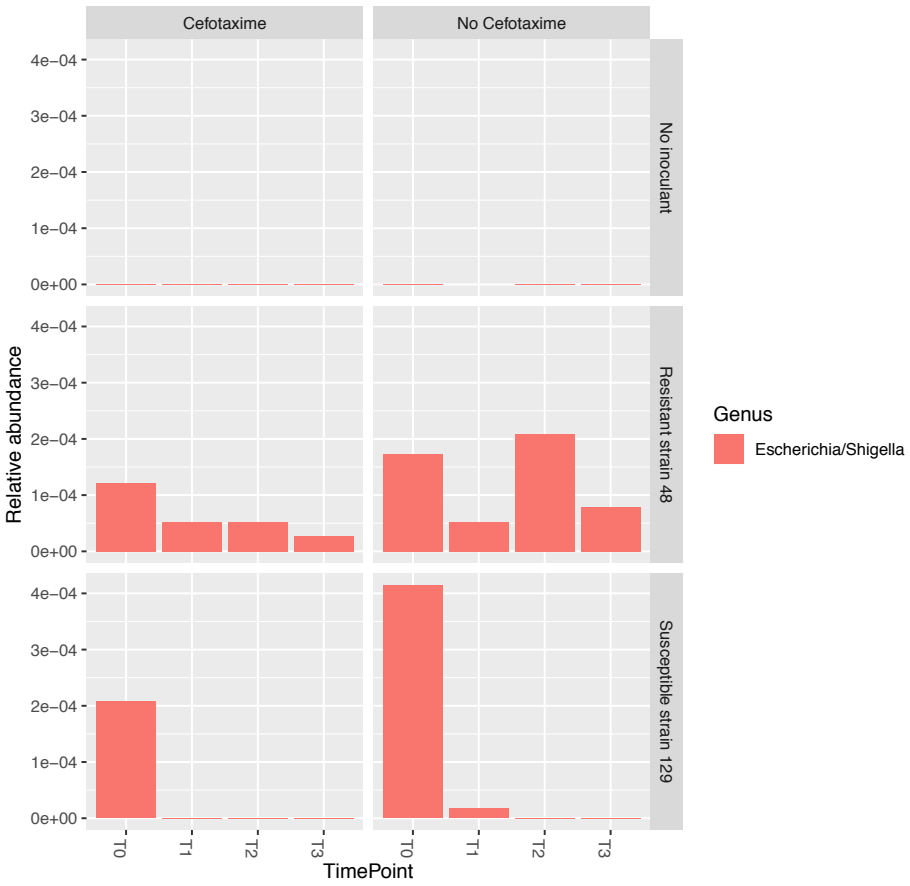


Figure 4.9 Detection of the genus *Escherichia* in the different treatments. *Escherichia* was detected at all the time points tested in the microcosms spiked with strain 48.

The *bla*$_{\text{CTX-M}}$ Group 1 was not detected in the control nor in the sample spiked with *E. coli* 129 by qPCR analysis (Figure 4.10 and 4.11).

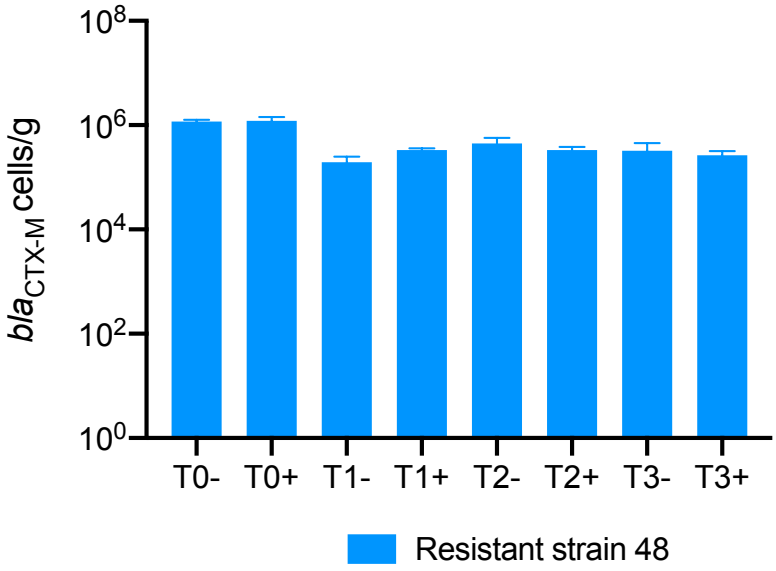


Figure 4.10 Abundance of the bla_{CTX} Group 1 in the microcosm spiked with *E. coli* 48.

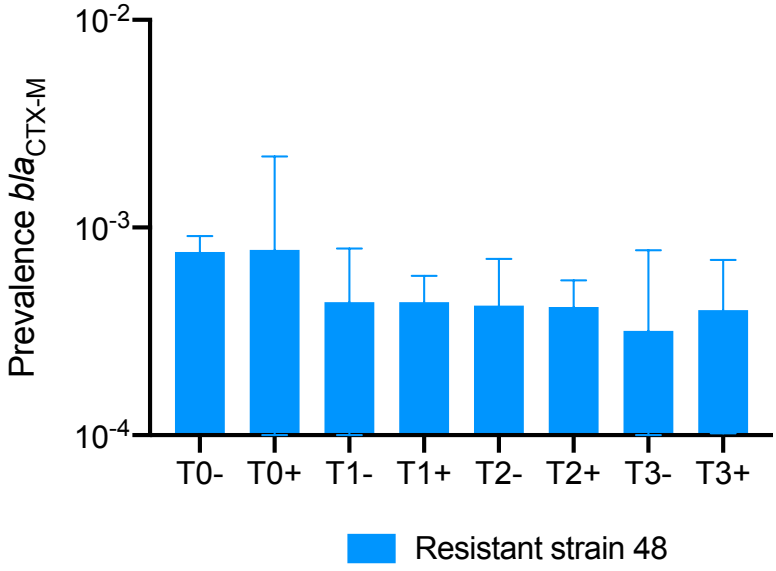


Figure 4.11 Gene prevalence for bla_{CTX} Group 1 in the microcosm spiked with *E. coli* 48.

The data indicates that *E. coli* 48 has significantly improved survival in the ABs compared to antibiotic susceptible non-pathogenic *E. coli* 129. An attempt to quantify the number of *E. coli* in the microcosm spiked with 48 was done for T1 and T2 using the Colisure kit. The biomass was diluted in PBS (dilution 1/200, 1/400, 1/1000) and for all dilutions Colisure was positive in all wells making the quantification impossible. Further dilution was not possible due to insufficiency of sample.

4.4 Discussion

In this study microcosms were used to simulate farm-based AD using slurry inoculum co-digested with FOG. This system was validated as evidenced by the production of various gases including methane. The analysis of DNA from these systems was successfully used to investigate the AD community diversity and survival of the *E. coli* inoculants in ABs. Even though the quality of the DNA was verified by Qubit measurement and considered as acceptable, the rarefaction curves indicated that the whole diversity was higher than expected and there were still new sequences recovered so the asymptote was not reached, and some members of the community were not represented.

The addition of *E. coli* did not affect the microbial communities, with Firmicutes and Bacteroidetes being the two most abundant phyla, confirming results previously found in ABs (Klocke *et al.*, 2007; Jaenicke *et al.*, 2011; Zhang *et al.*, 2011; St-Pierre and Wright, 2014). The Ruminococcaceae family, involved in the first step of the biomass digestion was identified in each microcosm (APPENDIX 8) and this is as expected for the first stage of cellulose digestion (Jaenicke *et al.*, 2011; Maus *et al.*, 2016). The importance of the phylum Firmicutes for the hydrolysis of cellulosic material in ABs is widely reported (Wirth *et al.*, 2012). The phylum Euryarchaeota was recovered despite the use of primers targeting eubacteria and this group are commonly found in ABs as they are responsible for methane production (Cardinali-Rezende *et al.*, 2009; Ritari *et al.*, 2012). The Euryarchaeota are a highly diverse phylum and the *Methanosarnica* genus, a methanogen which can transform acetate to methane in the last step in an AD was observed in all samples (APPENDIX 9 and 10) (Sun *et al.*, 2015). The headspace biogas was removed daily and production of methane was observed at day three. Taken together, these results showed evidence of the presence of a realistic digester community with the production of biogas in the small-scale bioreactors.

The addition of cefotaxime had an effect on the microbial communities notably on *Ruminiclostridum_1*, *Ruminococcus_1*, *Ruminofilibacter*, *Mariniphaga*, *Hydrogenispora*, *Ruminiclostridum*, *Ruminococcaceae_UCG-012*, *Caldicopracter* and *Herbinix*. The firmicutes (*Herbinix*, *Ruminiclostridum_1*, *Caldicopracter*, *Hydrogenispora*, *Ruminococcus_1* and *Ruminococcaceae_UCG-01*) were negatively impacted by the presence of cefotaxime which was unexpected as generally anaerobic

activity of cefotaxime is variable against members of this group such as species of the *Clostridium* genus. Members of the Bacteroidetes phylum were also negatively impacted in contrast to reports that in the gut Bacteroidetes are not affected by cefotaxime (Wexler, 2007). Whereas other firmicutes within the *Fastidiosipila* and *Gelria* were positively enriched in the presence of cefotaxime. Clearly little is known about the impacts of antibiotics on the AD microbiome.

The objective of this study was to determine the effectiveness of AD for reduction of the *E. coli* population and monitor the prevalence of the $bla_{\text{CTX-M}}$ gene. The microcosms were used to assess the ability of AD to remove pathogenic *E. coli.* At the genus level, *Escherichia* species were identified at each time point in the microcosm spiked with the pathogen 48 but the non-pathogenic commensal strain 129 was not detected using the 16S rRNA gene primers. Strain specific detection was not done because of the need to investigate unmodified naturally occurring *E. coli* strains. However, a species-specific qPCR was attempted using *uidA* gene encoding for the β-glucuronidase enzyme for *E. coli,* but this failed to work adequately due to background amplification. Clearly the key impact of this work is the significant survival of the ExPEC *E. coli* which has implications for the removal of pathogenic strains from ABs and this applies not only to agricultural digesters but also those used for sewage treatments (Estrada *et al.*, 2004; Pandey and Soupir, 2011; Beneragama *et al.*, 2013; Resende *et al.*, 2014). The culture-independent data proved that *E. coli* ST131 responsible for many UTIs survived AD whereas the commensal *E. coli* did not. In agreement with this, the qPCR data showed that $bla_{\text{CTX-M}}$ ARG persisted throughout the nine-day incubation. Culture-independent studies investigating the fate of ARGs during AD showed that temperature appeared to be a critical variable which meant that higher temperatures were more effective at removing ARGs (Ghosh *et al.*, 2009; Diehl and Lapara 2010). However the mesophilic microcosms used in the current study were effective in removing the strain 129 but not the *E. coli* ST131 which is a highly virulent strain responsible for the spread and dissemination of ARGs and of the $bla_{\text{CTX-M-15}}$ (Woodford *et al.*, 2009; Can *et al.*, 2015; Hertz *et al.*, 2016). The success of this clone ST131 is driven in part by the acquisition of VFs, such as genes providing resistance to third-generation cephalosporin ($bla_{\text{CTX-M-15}}$) but also adhesins and other VFs that may help in survival outside of the host. The inoculant strain 48, used here as a representative of the ST131 clone, was able to adapt to environmental challenges

such as AD and originally was isolated from the River Sowe where it was surviving in river sediment downstream of a WWTP (Hill, 2016). A better understanding of pathogen resilience outside the host will help to mitigate the spread of this strain (Pitout and DeVinney, 2017). The representation of the phylogenetic tree performed by Nabil-Fareed Alikhan using the EnteroBase and reference genome across *E. coli* showed the distinct position of strain 129 compared to strain 48 (Zhou *et al.*, 2019) (Figure 4.12). *E. coli* 48 carries two plasmids and these were found to contain both ARGs and virulence genes (Chapter 2). Some evidence suggests that such plasmids contribute also to the overall fitness of the host strain in addition to offsetting the impact of plasmid gene expression (Hall *et al.*, 2020). *Escherichia* species can survive in hostile environments under different stress conditions however 129 was surviving in the River Sowe where it was isolated from sediment but was vulnerable to AD. Bacterial load reduction during AD is the result of high level of volatile fatty acids and acidic pH; these conditions are toxic for some bacteria (Kunte *et al.*, 2004), but as 48 carried different resistant determinants and virulence genes in comparison to 129 might explain why *E. coli* are detected in the microcosms spiked with 48.

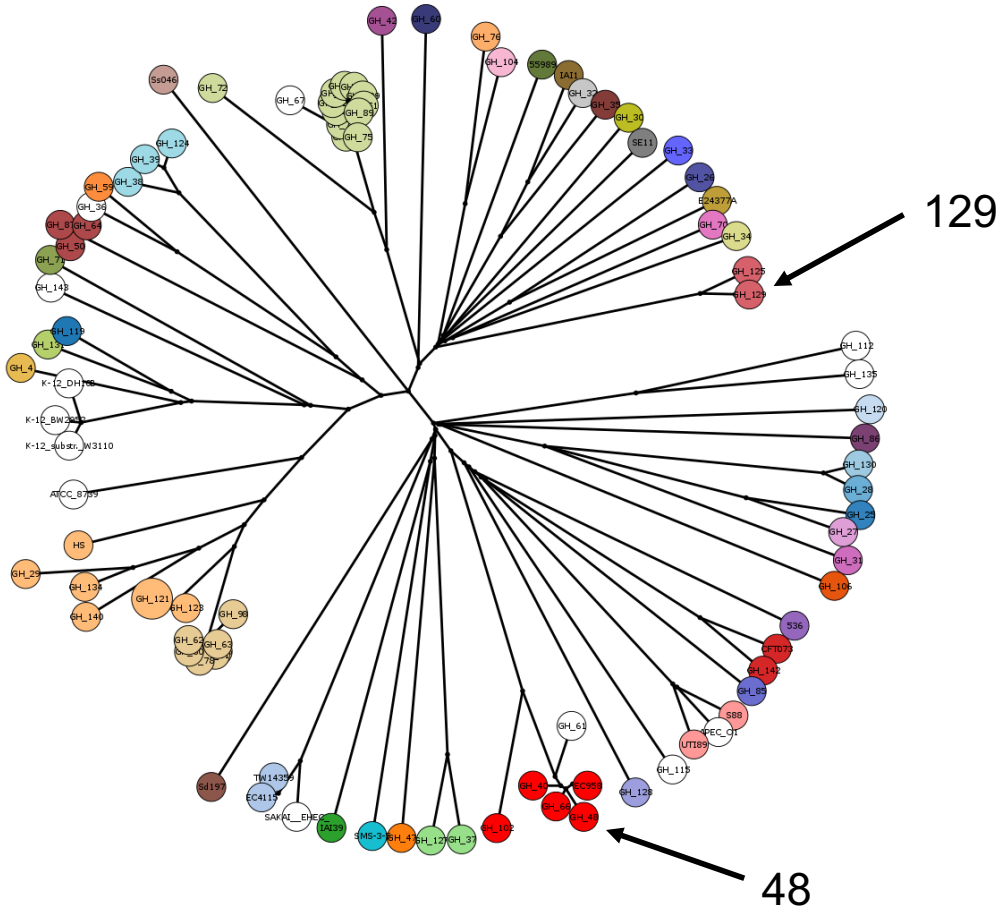


Figure 4.12 Phylogenic three using GrapeTree from EnteroBase using reference genomes across *E. coli* (Zhou *et al.*, 2019).

The evidence for persistence of ST131 in the current experiments was based on culture-independent methods and relied on the correlation of qPCR with the presence of intact cells. Viable *E. coli* were detected at day three which supported interpretation of persistence, but DNA can be cell-free (eDNA) resulting from the lysis of dead cells or active release by living cells. This eDNA is readily degraded by nucleases in microbial communities but can persist by adsorption or binding to organic material gaining protection from degradation (Agnelli *et al.*, 2004; Torti *et al.*, 2015). The persistence of eDNA depends on a number of factors such as DNA composition, methylation as well as the different environmental conditions such as temperature and pH (Pietramellara *et al.*, 2008). The DNA of both 129 and 48 was exposed to the same conditions and so would behave in a similar way. The disappearance of 129 DNA proves the strain is dead in contrast to the recovery of strain 48 DNA which proves it survives. Zhang *et al,* (2015) showed that different ARGs respond differently under

mesophilic or thermophilic digestion, with some of the ARGs cannot be reduced by either mesophilic or thermophilic conditions (Zhang *et al.*, 2015).

This experiment was performed for nine days and can be seen as a promising pilot study. Little is known about the removal of ARGs during the digestion, but this is an important issue as it controls the dissemination of AMR in the environment as the biomass can be used as fertiliser (Munir *et al.*, 2011). Nolan *et al.* (2018) emphasised the importance of developing a test system to evaluate the fate of pathogens during AD as the slurry is known to contain a range of bacteria that can cause diseases in animals (Nolan *et al.*, 2018). In addition to drug resistant *E. coli* there would be other mastitis-causing pathogens such as staphylococci. However few studies has investigated the diversity of those diverse groups (Sahlström, 2003; Dennehy *et al.*, 2018; Nolan *et al.*, 2018).

Chapter 5:

Final discussion and conclusions

The *E. coli* ST131 are responsible for ExPEC infections and this pathogenic clonal group can be excreted in the urine from UTIs and also in the faeces due to carriage in the GIT. Due to sewage disposal method, the group will frequently be discharged into rivers and the study reported in this thesis has demonstrated its ability to survive in diverse environmental locations. The group are often resistant to commonly used antibiotics in both community and hospital-acquired infections. Other attributes which could contribute to the survival of these strains in the environment relate to biocide resistant in the form of efflux pumps in addition to SOS responses which could provide resilience to UV and white light. In the current study all of these features were found on transferable plasmids which have been acquired through selection possibly in the clinic or the community and most likely relate to the acquisition of AMR. It is possible to posit that the evolution of a pathogenic strain results in a more robust group able to survive in the environment outside of the host. A combine hypothesis resulted from this work are summarised in Figure 5.1.

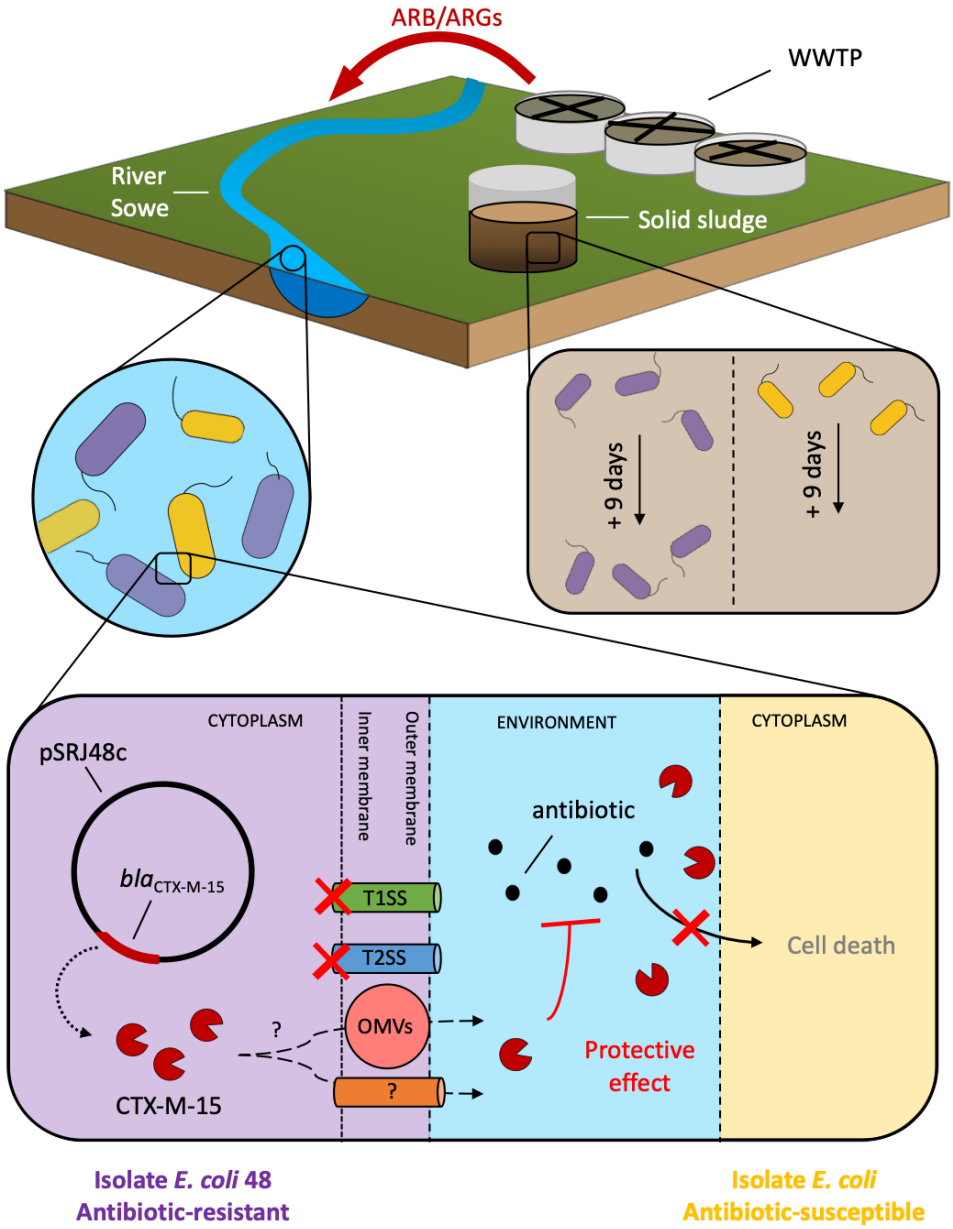


Figure 5.1 Schematic summary of the work developed in the current study. *E. coli* strain 48 was isolated from a river downstream of a WWTP and $bla_{CTX-M-15}$ was found on the pSRJ48c plasmid. Mechanism of secretion of the enzyme remains elusive but the secretion of CTX-M-15 provides a protective effect against cefotaxime for surrounding susceptible cells. *E. coli* strain 48 survived through a nine-day anaerobic incubation in contrast to the sensitive strain that failed to survive.

The primary aim of this research was to understand the secretory mechanism of an ESBL-producing *E. coli* ST131 strain 48, isolated in the UK water system, downstream of a WWTP and to develop a better understanding of $bla_{CTX-M-15}$ gene expression. To this end, a new bioinformatic method based on long-read sequencing (ONT) was developed to rapidly sequence, assemble and undertake primary annotation of a resistant plasmid associated with *E. coli* 48. This method allowed successful *de novo* assembly and characterisation of ARGs location, composition,

order and putative mechanism of transposition. Two plasmids were identified with the Inc group IncB/K/O/Z and IncFIB/IncFII, both were a mosaic of ARGs, MGEs, virulence and transfer genes. Surprisingly, and in contrast with the literature, *bla*$_{\text{CTX-M-15}}$ was the only ARG found on the IncB/K/O/Z, this gene was found in close proximity to IS elements. The gene could be mobilised to a new location in the same strain or *via* a new or the same MGE with transfer to a new host contributing to the dissemination of antibiotic resistance to clinically relevant pathogens (Carattoli, 2013). The two plasmids identified in this study can promote survival of the bacterium in the presence of antibiotics but also presumably impose a fitness cost when they enter a new host. General principles behind the fitness cost have now started to be identified but the specific molecular basis of these costs is not fully understood and it is still unknown what makes a specific host-plasmid association successful (San Millan *et al.*, 2014; Porse *et al.*, 2016). The current work has clearly demonstrated that both plasmids and host have evolved into a mutualistic association of benefits with the host providing the plasmids with a fit and highly competitive host environment. The majority of studies that investigated plasmid fitness costs have used laboratory bacterial strains and plasmids and avoided pathogenic bacteria such as *E. coli* or *Klebsiella pneumoniae* that are the most concerning cause of multi-resistant infection in hospital (Brusselaers *et al.*, 2011). More rigorous study of the host-plasmid association is needed to understand how fitness is achieved to compensate for plasmid carriage and constitutive gene expression. A key observation in the current study was that plasmid genes were constitutively expressed. The general consensus is that plasmids that are intermittently beneficial and costly to the host do not persist and are lost over time or integrated into the host chromosome (Harrison and Brockhurst, 2012; MacLean and San Millan, 2015). Evidence showed persistence over time, and this persistence whether the plasmid is costly or not is called the "plasmid paradox"(Harrison and Brockhurst, 2012). The cost of the plasmid carriage can be compensated by mutations to either the plasmid or the host (Harrison and Brockhurst, 2012). These studies have all focused on a strain of *Pseudomonas* genus (San Millan *et al.*, 2014; Harrison *et al.*, 2015; San Millan *et al.*, 2015; Harrison *et al.*, 2016). No such study on fitness has ever been done on *E. coli* pathogenic strains such as ExPEC ST131. However, it was clear from the work described in Chapter 2 that the resistant plasmid in *E. coli* 48 were transferable and in the case of pSRJ48c could be conjugated into other *E. coli* strains. This particular plasmid carried the key gene *bla*$_{\text{CTX-M-15}}$ which

also has been widely disseminated and could contribute to improved fitness of the host.

On the basis of previous studies describing the secretory mechanism of NDM-1 and CTX-M-1, an investigation was done to gain a better understanding of the enzyme secretion and its cellular location. The NDM-1 enzyme was secreted outside of the cells which in an *E. coli* host is in contrast to many other β-lactamases. It was hypothesized that CTX-15 was also secreted outside the cells. Clearly there is some fitness benefit to the acquisition of both of these genes as they have spread across the world in a short period of time (Rossolini *et al.*, 2008; Hawkey and Jones, 2009; Canton *et al.*, 2012). Exo- and whole-cell proteomic studies identified CTX-M-15 as the major secreted ESBL confirming *in silico* analysis (this was first presented at a The International Society for Plasmid Biology meeting 2016 in Cambridge). Expression of the $bla_{\text{CTX-M-15}}$ in another *E. coli* strain confirmed that this gene was responsible for the protective effect against cefotaxime (Figure 5.1). Despite being unable to demonstrate the secretion system involving this enzyme, it was demonstrated that CTX-M-15 was the major ESBL secreted in *E. coli* 48. It is feasible that secretion of CTX-M-15 represents an evolutionary advantage, no damage would occur to the cell-wall if the antibiotic is disabled outside of the cell in opposition to hydrolysis in the periplasm. In addition, the secreted β-lactamase provided a protective effect to other bacteria against antibiotics. It is not clear what the evolutionary benefit of this aspect of secretion might be, but the effect would be to allow sensitive bacteria to survive within the population which could be a benefit to resistant bacteria. An analogy can be made with the refuge strategy used in crops production in order to reduce the selective pressure and delay the spread of insect resistant; havens for non-resistant insects is supplied (Tabashnik *et al.*, 2008) (Figure 5.2).

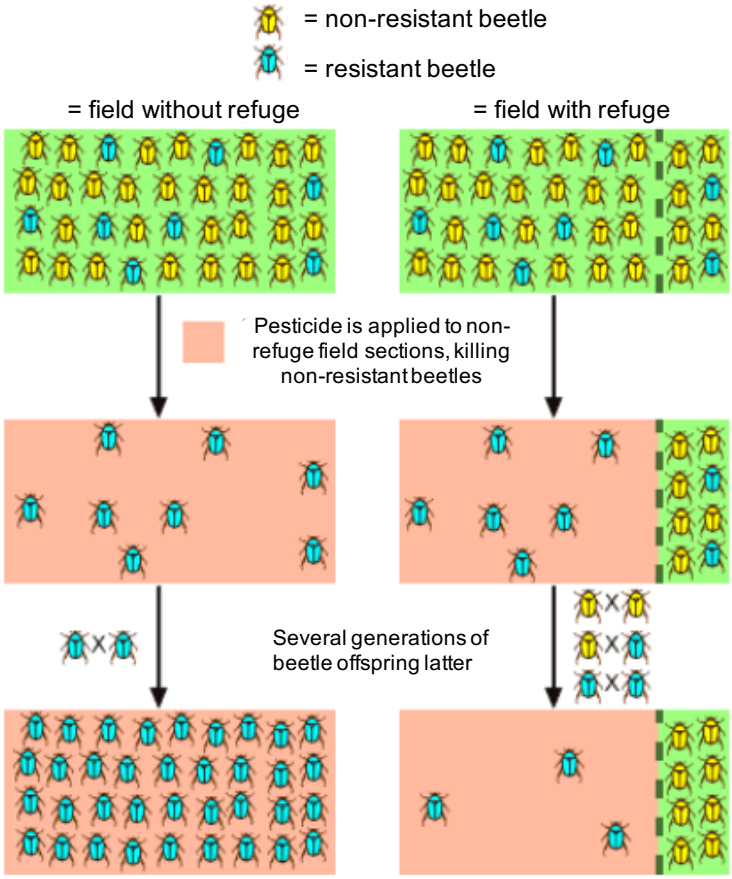


Figure 5.2 Concept of refuge area, used to mitigate the spread of resistance. Figure is extracted from (Evolution, accessed 16 June 2020).

It was evident from Chapter 2 and 3 that the mechanism of secretion was transferred with the *bla*$_{\text{CTX-M-15}}$ but no clear mechanism could be identified. *In silico* analysis indicated the presence of signal peptide but no secretory system could be pinpointed as the mechanism of export with experiments ruling out T1SS and T2SS. Given that the mechanism identified in the secretion of NDM-1 was vesicular it is possible that the same mechanism has been adapted for CTX-M-15. This was implied in a recent study published by a Korean group who investigated the secretion of CTX-M-1, although cytoplasmic leaking may have occurred during their experiment (Kim *et al.*, 2018). Therefore, it is reasonable to assume that all transfer involving pSRJ48c will provide new hosts with a mechanism of β-lactamase secretion (a combination of signal peptide and other parameters as it does not happen with TEM). The key feature of AMR is the transferability and rapid acquisition of resistant traits which can occur *in vitro*, *in vivo* and *in situ.* No experiments have been done to determine if strain 48 was capable of conjugation in the river. Investigations that involved environmental isolates

are more likely to give a transfer rates occurring in the environment however it is challenging to replicate mating conditions in a laboratory due to the various parameters such as pH, temperature or oxygen availability and if those factors affect the metabolic activity of the donor they will also affect the transfer rate (Smalla *et al.*, 2015).

The final aspect developed in this thesis was to evaluate the effectiveness of AD on $bla_{\text{CTX-M-15}}$ gene prevalence and to compare survival of *E. coli* 48 with a non-resistant commensal bacterium (*E. coli* 129) in the presence of sub-lethal concentration of cefotaxime. *E. coli* 48 was isolated downstream of a WWTP but it remained uncertain whether the strain was growing or surviving. The attempt to simulate ABs revealed the survival of the ExPEC *E. coli* 48 and the persistence of $bla_{\text{CTX-M}}$ through a nine-day anaerobic incubation. This was a stark contrast to the commensal strain *E. coli* 129 which died after three days. This finding has a key impact for the removal of pathogenic strains from agricultural digesters and sewage treatments and without an appropriate biocidal action can cause potential threats to human health (Devarajan *et al.*, 2015; Su *et al.*, 2015). Studies have shown a decrease in the relative abundance of pathogenic microorganisms after AD of sewage sludge, but different species might have a different susceptibility to AD (Sahlstrom *et al.*, 2008; Ju *et al.*, 2016). Temperature, pH, ammonia concentration, volatile fatty acids and solid retention time are important factors for pathogen deactivation, but the entire mechanism has not been fully understood. The use of digestate as fertiliser may pose a potential risk as it can be a carrier of pathogens with ARGs and specific pathogens can transfer their genes in the soil (Manser *et al.*, 2015; Wang *et al.*, 2015; Zhao and Liu, 2019). The EU regulations stipulate that digestates are suitable for use if the level of *E. coli* is below 100/g, but it is important to note that even if this level is low it does not mean the absence of a potential pathogenic risk due to the occurrence of highly resistant strain with MGEs (Ahn *et al.*, 2007). Li *et al.* (2015) showed that some pathogens can be enriched, or some can emerge or re-emerge after AD (Li *et al.*, 2015). A comprehensive survey on pathogen content in AD needs to be done to evaluate survival and could lead to optimisation of the process to minimise risks. The survival of 48 poses multiple risks as this strain can be the host for other ARGs, but it can also spread ARGs *via* its MGEs such as plasmids, transposons or integrons. Characterisation of the digester microbial communities has shown a high abundance

of Firmicutes and Bacteroidetes in the ABs during the nine-day experiment. This observation indicates a risk as Bacteroidetes can be considered as potential pathogens because they can harboured ARGs (Stiborova *et al.*, 2015; Sun *et al.*, 2016). This study highlighted questions around the suitability of AD for pathogens controls and reduction.

In conclusion, the role of environmental contamination in the transmission of *Enterobacteriaceae* and in particular *E. coli* ST131 is increasingly recognized. However, factors influencing the duration of survival in the environment have not yet been extensively studied. Future investigations should be undertaken to study the ecological factors that make certain STs such as ExPEC very successful pathogen which can survive in hostile environment outside of the host.

Further work:

- Determine the stability of *bla*$_{\text{CTX-M-15}}$ plasmid during prolonging growth in strain 48 but also in laboratory strains.
- Study of the fitness cost of the pSRJ48c plasmid in the host bacterium.
- Investigate the effect of the double KO of T1SS and T2SS on the secretion of CTX-M-15.
- Use of culture-based techniques on selective media to investigate the survival of *E. coli* ST131 strain 48 during AD.
- Study of the level of expression of *bla*$_{\text{CTX-M-15}}$ during AD using RT-qPCR.

APPENDIX 1

Appendix 1 ORFs identified in pSRJ48c.

Coding region	Length (bp)	Function encoded
EutE	501	Ethanolamine utilization protein EutE
traY	2160	Conjugative transfer: oriT nicking protein TraY
traX	570	F pilin acetylation
	1206	Conjugal transfer protein
traV	621	Conjugal transfer protein TraV
	3045	Conjugal transfer protein
orf7	546	Hypothetical protein
orf8	381	Hypothetical protein
orf9	360	Hypothetical protein
orf10	627	Conjugal transfer protein TraT
traS	252	Surface exclusion porteinTraS
traR	399	Conjugal transfer protein TraR
TraQ	531	Conjugal transfer protein TraQ
trap	714	Conjugal transfer protein TraP
traO	1332	Conjugal transfer protein TraO
traN	975	Conjugal transfer protein TraN
traM	696	Conjugal transfer protein TraM
	351	Conjugal transfer protein
	4062	DNA primase
	291	Conjugal transfer protein
traJ	1149	Plasmid transfer ATPase TraJ
	837	Conjugal transfer protein
traH	459	TraH protein
traF	1203	Conjugal transfer protein TraF
traE	822	Conjugal transfer protein TraE
	1362	Shufflon system plasmid conjugative transfer pilus tip adhesin PilV
	627	Prepilin peptidase
	486	Lytic transglycosylase
	537	Prepilin-type cleavage/methylation domain-containing protein
	1095	Type II secretion protein F
	1509	Type II secretion protein E
	459	Type IV pilus biogenesis protein PilP
	1296	Pilus assembly protein
	1620	PilN family type IVB pilus formation outer membrane protein
	438	pilM
	1071	Pilus assembly protein
	243	Pilus assembly protein PilI

	1695	Flotillin
orf39	621	hypothetical protein
traC	663	F pilus assembly protein TraC
nusG	642	transcription termination factor NusG
repA	879	incFII family plasmid replication initiator RepA
copG	279	ribbon-helix-helix protein, CopG family
orf44	276	Type II toxin-antitoxin system RelE/ParE family toxin
orf45	276	Hypothetical protein
orf46	267	Hypothetical protein
yafA	156	Hypothetical protein
	603	proQ/FINO family protein
orf49	1053	Hypothetical protein
orf50	591	Hypothetical protein
orf51	255	Hypothetical protein
orf52	414	Hypothetical protein
	780	ResD
orf54	645	Hypothetical protein
orf55	309	Hypothetical protein
parM	981	Plasmid segregation protein parM
	417	Plasmid stability protein
umuC	1275	Translesion error-prone DNA polymerase V subunit UmuC/ UmuC UV protection protein
impA	438	UV protection and mutation protein
impC	249	UV protection and mutation protein
orf61	927	Hypothetical protein
yhdJ	684	Putative methylase
yceA	222	Hypothetical protein
orf64	435	Hypothetical protein
ychA	771	Hypothetical protein
	426	Antirestriction protein
	423	Hypothetical protein
	192	p035
ssb	528	Single-stranded DNA-binding protein
ykfF	234	Hypothetical protein
parB	1959	ParB-like partitioning protein
psiB	438	Plasmid SOS inhibition protein B
psiA	720	Plasmid SOS inhibition protein A
ygaA	597	Hypothetical protein
ard	501	Anti-restriction protein
ondC	435	Post-segregation killing protein PndC
ydfB	267	Hypothetical protein
orf78	390	Hypothetical protein
tnpA	915	Transposase

yhgA	183	Hypothetical protein YhgA-like transposase
	252	XRE family transcriptional regulator
ydiA	849	Hypothetical protein YdiA
	336	Molybdopterin-guanine dinucleotide biosynthesis protein MobC
nikA	333	Relaxosome component protein
nikB	2712	Relaxase NikB
tolA	396	tolA family protein
orf87	150	Hypothetical protein
orf88	294	Hypothetical protein
trbC	2304	Conjugal transfer protein TrbC
	1125	Protein-disulfide isomerase
trbA	1299	Conjugal transfer protein TrbA
orf92	273	Hypothetical protein
$bla_{\text{CTX-M-15}}$	876	β-lactamase resisant
orf94	123	Hypothetical protein
tnpA	1098	Transposase
tnpA	981	Transposase of IS5/IS1182 family
orf97	177	Hypothetical protein
	468	Thermonuclease
orf99	681	Hypothetical protein
orf100	252	Hypothetical protein
orf101	216	Hypothetical protein
orf102	369	Hypothetical protein
orf103	243	Hypothetical protein
pndA	153	Postsegregation killing system, counter protein for PndA
	513	RNA polymerase subunit sigma

APPENDIX 2

Appendix 2 ORFs identified in pSRJ48t.

Coding region	Length (bp)	Function encoded
crcB	381	Putative membrane protein
sitD	858	Transportation of Fe, Mn
sitC	858	Transportation of Fe, Mn
sitB	828	Transportation of Fe, Mn
sitA	915	Transportation of Fe, Mn/
orf6	291	Hypothetical protein
umuC	225	DNA polymerase V subunit
tnpA_1	357	Putative transposase
repA	978	RepFIB replication protein RepA
orf10	120	Hypothetical protein
IntI1	741	Integrase
hylF	1110	Hemolysin/ Creating of pores in membranes of host cells (cell lysis)
ompT	954	Outer membrane protease
orf14	150	Hypothetical protein
orf15	264	Hypothetical protein
	327	IS629 transposase
	888	IS629 transposase
orf18	174	Hypothetical protein
	291	IS150 protein InsAB
estA	1182	ABC transporter, efflux pump protein
estB	1941	ABC transporter, ATP-binding protein
orf22	189	Hypothetical protein
orf23	228	Hypothetical protein
orf24	942	Hypothetical protein
orf25	387	Hypothetical protein
orf26	111	Hypothetical protein
	867	Putative integrase
	426	IS66 family transposase
	351	IS66 family transposase
	1614	IS66 family transposase
	1194	GTPase
tnpA	906	Transposase of IS2
tnpA	366	Transposase of IS2
borD	294	bacteriophage lambda Bor protein
orf35	183	Hypothetical protein
orf36	291	Hypothetical protein
	636	Putative integrase
iroB	1116	Putative glucosyltransferase
iroC	3770	ATP binding cassette (ABC) transporter-like protein
ironD	1230	Putative ferric enterochelin esterase
ironE	957	Putative hydrolase
iroN	2178	Siderophore receptor, use of Fe ions obtained from the body host
orf43	216	Hypothetical protein
orf44	291	Hypothetical protein
	561	Putative transmembrane protein
orf46	159	Hypothetical protein
yadA	483	
yacC	849	Putative exoribonuclease
yacB	279	Putative plasmid stabilization system
yacA	255	Putative repressor
orf51	138	Hypothetical protein

tnpA	411	Transposase of IS2
tnpA	909	Transposase of IS2
cvaA	1275	Colicin V secretion protein
cvab	2097	Colicin V secretion/processing ATP-binding protein
cvaC	312	colicin V synthesis protein
	228	Putative transmembrane protein
orf58	132	Hypothetical protein
orf59	282	Hypothetical protein
cbi	528	colicin B immunity protein Cbi
cma	816	colicin M immunity protein Cmi
cmi	354	colicin M activity protein Cma
orf63	291	Hypothetical protein
tnpA	351	Transposase of ISEC23
tnpA	222	Transposase of IS66
tnpA	186	Transposase of IS66
argR	477	
arcD	1404	
arcB	1005	
arcA	1221	
cdiA4	246	Deoxyribonuclease
RepA1	429	Replication initiation protein RepA1 of FII replicon
RepA2	258	Negative regulator of repA1 expression, FII replicon
snrB'	150	SrnB' toxin
orf75	183	Hypothetical protein
orf76	291	Hypothetical protein
fino	561	FinO fertility inhibition protein
traX	573	F pilin acetylation
traI	1494	OriT nicking and unwinding protein TraI
traI	2769	OriT nicking and unwinding protein TraI
traD	729	TraD conjugative protein
tnpA	2898	Transposase
tnpR	297	Resolvase
tnpR	315	Resolvase
traD	2781	OriT nicking and unwinding protein TraI
traV	516	F pilus assembly protein TraV
trbG	252	Conjugal transfer protein TrbG
trbD	162	Conjugal transfer protein TrbD
trbD	138	Conjugal transfer protein TrbD
traP	591	Putative conjugal transfert protein TraP
traB	1428	F pilus assembly protein TraB
traK	729	F pilus assembly protein TraK
traE	567	F pilus assembly protein TraE
traL	312	Conjugal transfer pilus assembly protein TraL
traA	366	Fimbrial protein precursor TraA (Pilin)
traY	216	Conjugative transfer: oriT nicking protein TraY
traJ	648	Positive regulator of conjugal transfer operon, protein TraJ
traM	384	Conjugal protein TraM
yubQ	510	Putative X polypeptide (P19 protein)
yubP	822	
orf102	288	Hypothetical protein
orf103	237	Hypothetical protein
ydcA	564	putative adenine-specific DNA methylase
ydbA	1362	Hypothetical protein
ydeB	231	Hypothetical protein
orf107	258	Hypothetical protein
orf108	183	Hypothetical protein
orf109	372	Hypothetical protein

yubl	426	Putative antirestriction protein
tnpA	972	Transposase of ISEC23
orf112	213	Hypothetical protein
orf113	577	Hypothetical protein
	339	putative transmembrane protein
sopB	972	Partitioning protein SopB
sopA	1167	Partitioning protein SopA
orf117	291	Hypothetical protein
tnpR	705	Resolvase
mcjD	1743	
mcjC	1542	
mcjB	627	
mcjA	177	
tnpA	705	Transposase
dfrA17	474	Trimethoprim resistant
IntI2	1014	Class 2 Integron
pinE	216	Prophage e14
tnpA	705	Transposase
tnpA	1437	Tranposase of Tn21 transposon
orf129	297	Hypothetical protein
orf130	1005	Hypothetical protein
orf131	177	Hypothetical protein
sul2	816	Sulfonomide resistant
aph(3")-lb	804	Aminoglycoside resistance
aph(6)-id;	837	Aminoglycoside resistance
orf135	540	Hypothetical protein
bla_{TEM}	861	β-lactamase resistant
tnpA	639	Tranposase
orf138	213	Hypothetical protein
orf139	258	Hypothetical protein
	447	Putative signal peptide protein
tnpA	330	Tranposase of IS2
tnpA	921	Tranposase of IS2
traC		
repE	252	Replication protein RepA
resD	783	Resolvase protein ResD
ccdB	306	Plasmid maintenance protein, toxin component
ccdA	219	Plasmid maintenance protein, antitoxin component
orf148	690	Hypothetical protein
orf149	690	Hypothetical protein
vagC	231	Virulence-associated protein VagC
vagD	417	Virulence-associated protein VagD
	2139	ABC transporter nucleotide-binding protein
vagC	378	Virulence-associated protein VagC
vagD	417	Virulence-associated protein VagD
estC	1308	ABC transporter, outer membrane component
orf156	1566	Hypothetical protein
orf157	1023	Hypothetical protein
orf158	156	Hypothetical protein
orf159	291	Hypothetical protein
iutA	2199	Ferric aerobactin receptor precursor LutA
iucD	1278	L-Lysine 6-monooxygenase
iucC	405	Aerobactin siderophore biosynthesis protein LucC
iucC	1350	Aerobactin siderophore biosynthesis protein LucC
iucB	948	N(6)-hydroxylysine acetylase LucB
iucA	1725	Aerobactin siderophore biosynthesis protein
orf166	504	Hypothetical protein

APPENDIX 3

Appendix 3 ARGs and efflux pump identified on the chromosome of strain 48.

Coding region	Length (bp)	Function encoded
ampH	842	ampC-type B-lactamase
acrA	1193	Antibiotic efflux pump
kdpE	677	Involved in potassium homeostasis
msbA	1748	Antibiotic efflux pump
marA	383	Antibiotic efflux pump
KpnF	329	Antibiotic efflux pump
	365	Antibiotic efflux pump
aac(3)-IIc	503	Aminoglysocides resistance
bla_{OXA-1}	830	β-lactamase resistance
aac(6')-Ib	599	Aminoglysocides resistance
tet(A)	1199	Antibiotic efflux pump
ugd	1166	Resistance to cationic antimicrobial peptides
baeS	1403	Antibiotic efflux pump
YojI	1643	Antibiotic efflux pump
PmrF	968	Antibiotic efflux pump
emrK	1163	Antibiotic efflux pump
evgA	614	Antibiotic efflux pump
evgS	3593	Antibiotic efflux pump
emrR	530	Antibiotic efflux pump
emrA	1172	Antibiotic efflux pump
bacA	821	Resistance to bacitracin
AcrS	662	Antibiotic efflux pump
AcrE	1157	Antibiotic efflux pump
CRP	632	Antibiotic efflux pump
gadW	728	Antibiotic efflux pump
gadX	824	Antibiotic efflux pump
mdtO	2051	Antibiotic efflux pump
mdtN	1031	Antibiotic efflux pump
eptA	1643	Decreased binding of polymyxin B
gyrA	2627	Fluoroquinolone resistant
GlpT	1358	Resistance to fosfomycin
PtsI	464	antibiotic-resistant ptsI phosphotransferase
parC	2258	Resistance to fluoroquinolone
EF-Tu	1184	Resistance to elfamycin
UhpT	1325	Resistance to fosfomycin
acrR	647	Antibiotic efflux pump
marR	242	Antibiotic efflux pump
soxS	323	Antibiotic efflux pump
soxR	464	Antibiotic efflux pump

APPENDIX 4

Amino Acid Sequences

> CTX-M-15 [Strain 48]

MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGV
ALINTADNSQILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKK
SDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVT
AFARQLGDETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGD
SQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGSGGYGTTNDIAVIWP
KDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL

> TEM [Strain 48]

MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSG
KILESFRPEERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYS
PVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGD
HVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLID
WMEADKVAGPLLRSALPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVI
YTTGSQATMDERNRQIAEIGASLIKHW

> OXA [Strain 48]

MLAVKIKPFTKPILIMKNTIHINFAIFLIIANIIYSSASASTDISTVASPLFEGTEG
CFLLYDASTNAEIAQFNKAKCATQMAPDSTFKIALSLMAFDAEIIDQKTIFK
WDKTPKGMEIWNSNHTPKTWMQFSVVWVSQEITQKIGLNKIKNYLKDFDY
GNQDFSGDKERNNGLTEAWLESSLKISPEEQIQFLRKIINHNLPVKNSAIENTI
ENMYLQDLDNSTKLYGKTGAGFTANRTLQNGWFEGFIISKSGHKYVFVSAL
TGNLGSNLTSSIKAKKNAITILNTLNL

APPENDIX 5

Appendix 5 Exponential growth rate for *E. coli* BW+, *E. coli ΔtolC*+ and *E. coli ΔgspD*+. (t-Test; p=0.05).

	Cefotaxime (μg/ml)	Exponential growth rate: replicate			*p*-value
		1	2	3	
E. coli BW+	0	0.0289	0.0289	0.0242	/
	8	0.0254	0.0232	0.0232	2.28×10^{-1}
	16	0.0281	0.0288	0.0271	3.36×10^{-1}
	32	0.0245	0.0252	0.0252	4.30×10^{-1}
E. coli ΔgspD+	0	0.0318	0.0274	0.0281	/
	8	0.0224	0.0217	0.0205	**1.43×10^{-2}**
	16	0.0308	0.0273	0.0278	8.03×10^{-1}
	32	0.0308	0.03	0.0267	9.73×10^{-1}
E. coli ΔtolC+	0	0.0316	0.0345	0.0324	/
	8	0.0231	0.0205	0.0218	**6.49×10^{-4}**
	16	0.0252	0.0264	0.0200	**2.50×10^{-2}**
	32	0.0193	0.0152	0.0134	**3.24×10^{-3}**

Significant p-values are denoted in bold.

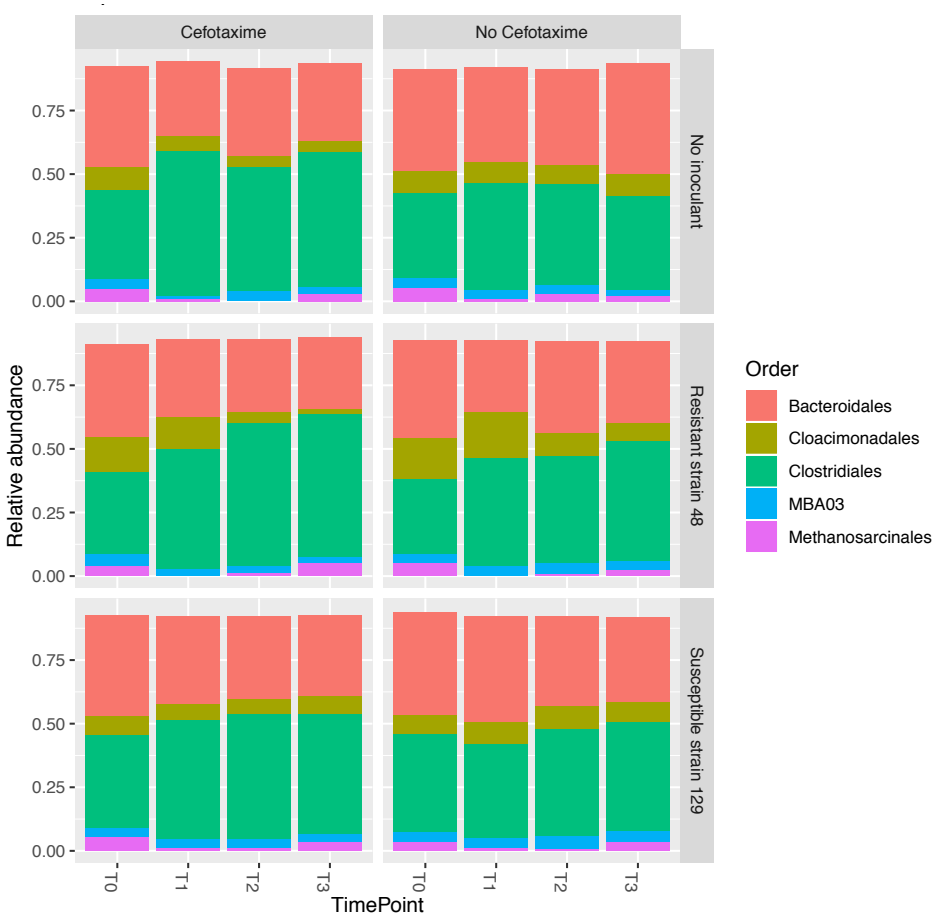
APPENDIX 6

Appendix 6 Exponential growth rate for strain 33 in presence of CM *ΔgspD*+ and CM *ΔtolC*+ (t-Test; p=0.05).

	Cefotaxime (μg/ml)	Exponential growth rate: replicate			*p*-value
		1	2	3	
E. coli BW+	0	0.0191	0.0175	0.0125	
	8	0.0166	0.0167	0.0128	7.01×10^{-1}
	16	0.0105	0.0222	0.0257	5.80×10^{-1}
	32	0.0288	0.0233	0.0245	**2.42×10^{-2}**
E. coli ΔgspD+	0	0.0134	0.0200	0.0290	
	8	0.0142	0.0094	0.0271	6.05×10^{-1}
	16	0.0104	0.0118	0.0129	1.85×10^{-1}
	32	0.0091	0.0105	0.0129	1.66×10^{-1}
E. coli ΔtolC+	0	0.0377	0.0331	0.0351	
	8	0.0085	0.0076	0.0101	**4.08×10^{-4}**
	16	0.0092	0.0102	0.0094	**2.81×10^{-3}**
	32	0.0114	0.0106	0.0106	**3.08×10^{-3}**

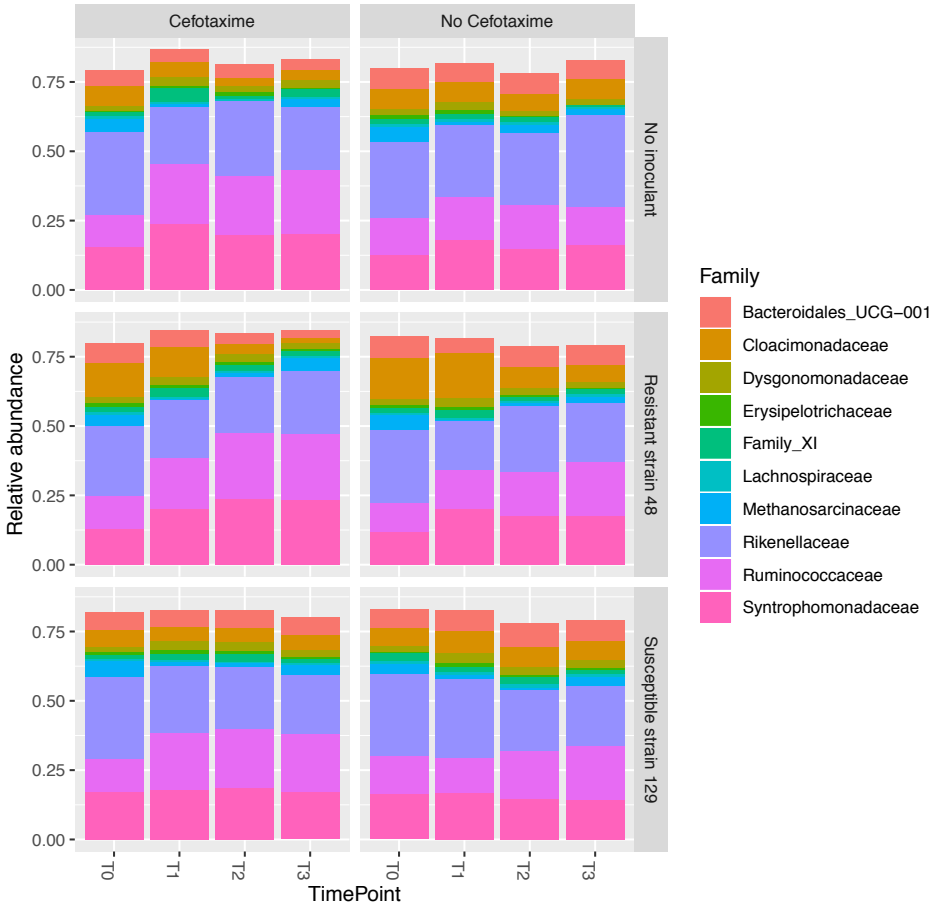
Significant p-values are denoted in bold.

APPENDIX 7



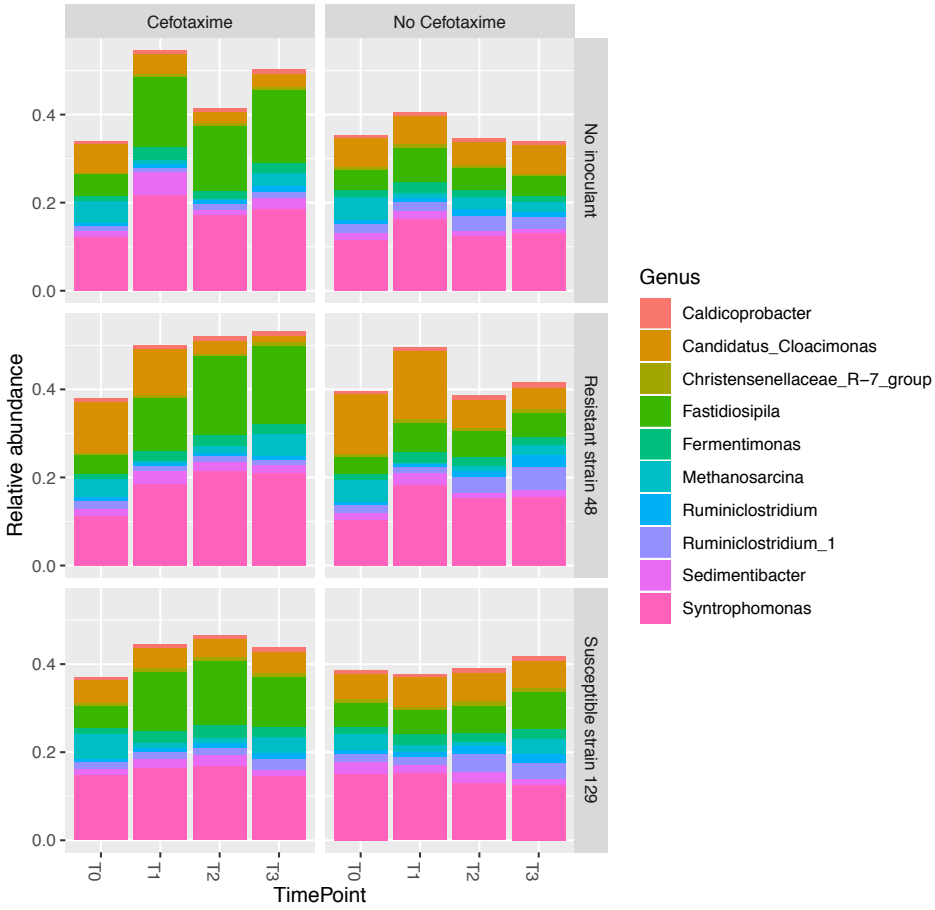
Appendix 7. The dominant order for each sample at the 4 times points tested based on 16S rRNA gene annotation using Silva. Selected Archaea were also represented.

APPENDIX 8



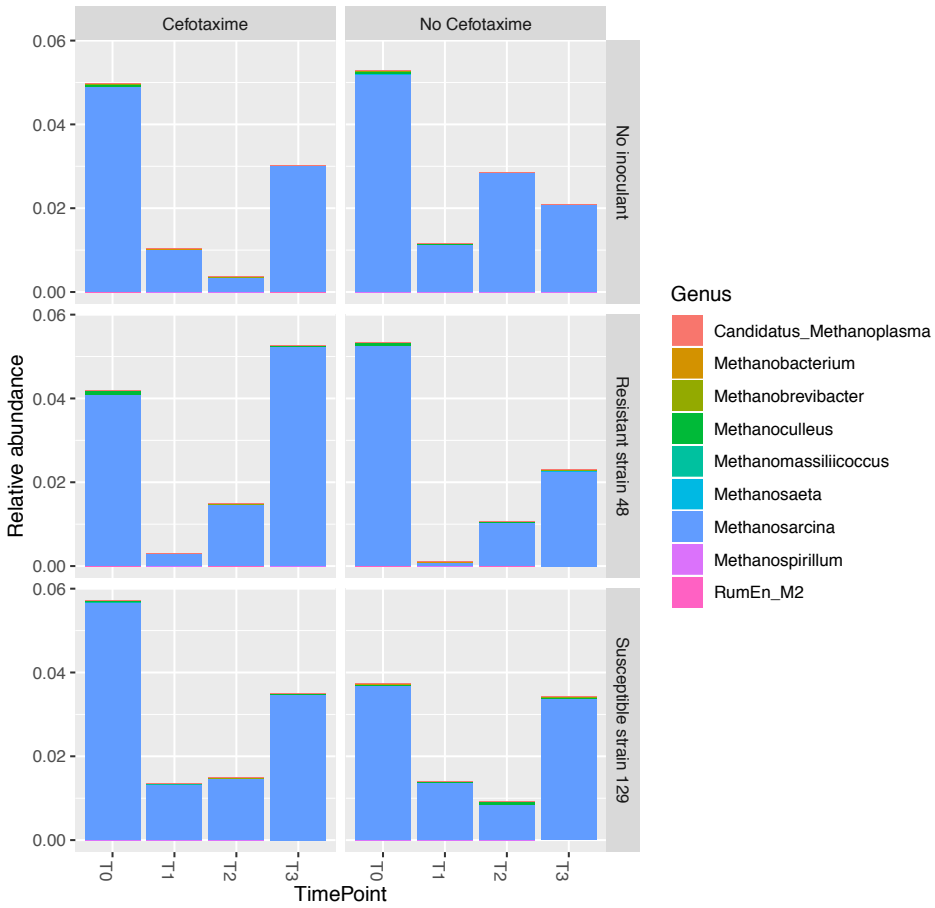
Appendix 8. The dominant family for each sample at the 4 times points tested based on 16S rRNA gene annotation using Silva. Selected Archaea were also represented.

APPENDIX 9



Appendix 9. The dominant genera for each sample at the 4 times points tested based on 16S rRNA gene annotation using Silva. Selected Archaea were also represented.

APPENDIX 10



Appenxdix 10. The dominant Archaea genera for each sample at the 4 times points tested based on 16S rRNA gene annotation using Silva.

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